AD_____

Award Number: DAMD17-97-1-7169

TITLE: The Identification of Genes Mediating Chemo-Sesitivity in Human Mammary Cells

PRINCIPAL INVESTIGATOR: Dale Haines, Ph.D.

CONTRACTING ORGANIZATION: Temple Universtiy School of Medicine
Philadelphia, Pennsylvania 19140

REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (1 Jun 97 - 1 Jun 00)

4. TITLE AND SUBTITLE
The Identification of Genes Mediating Chemo-Sesitivity in Human Mammary Cells

5. FUNDING NUMBERS
DAMD17-97-1-7169

6. AUTHOR(S)
Dale Haines, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Temple Universtiy School of Medicine
Philadelphia, Pennsylvania 19140

E-MAIL:
dhaines@astro.ocis.temple.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Our working hypothesis is that loss of function of a number of diverse checkpoint molecules will lead to the proliferation of chemo-resistance tumor cells. Besides the p53 pathway, relatively little is known regarding other checkpoint molecules whose loss of function results in chemo-resistance. This is probably due to the fact that loss of function of these molecules occurs through very subtle genetic alterations which are not easily detected by any current molecular techniques. Research being performed under this award is focusing on the identification of genes whose loss of function results in the proliferation of malignant epithelial cells that are resistant to the cytotoxic action of taxol, a microtubule formation inhibitor that is used in the treatment of breast cancer. This is being performed with a novel system based on a recently described technique (8) that allows for the isolation of genes encoding selectable recessive phenotypes. Identifying molecular pathways that are altered in chemo-resistance tumor cells will be critical for the future design of novel therapeutic strategies restoring chemo-sensitivity to chemo-resistant breast tumor cells.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 6/12/00 Date

Table of Contents

INTRODUCTION

Circumventing drug resistance is a critical step to increase the treatment success rate for women with breast cancer. The object of this grant is to improve our understanding of cellular genes whose loss of function results in the emergence of chemo-resistant tumor cells. It is becoming apparent that loss of function of checkpoint molecules regulating cell cycle progression and apoptosis are important in both tumor cell evolution and in the emergence of chemo-resistant malignant cells. Of the relatively small number of checkpoint molecules identified at this time, the best understood is the p53 tumor suppressor. Loss of p53 function through targeted deletion (in the case of the p53 knock-out mouse) renders cells deficient in their ability to undergo cell cycle apoptosis upon exposure to DNA damaging agents (1,2). Consistent with these in vitro observations, patients with hematological malignancies (3) and cancers of the stomach (4), lung (5) and breast (6) are less likely to respond successfully to chemotherapeutic agents as compared to patients with wild type p53 alleles. It is however obvious that p53 gene alterations do not explain all cases of chemo-resistance in human malignancies, as well as in breast cancer patients. Also, there is in vitro evidence which shows that breast tumor cells can become resistant to the action of chemotherapeutic drugs in the absence of p53 gene mutations (7).

Our working hypothesis is that loss of function of a number of diverse checkpoint molecules will lead to the proliferation of chemo-resistance tumor cells. Besides the p53 pathway, relatively little is known regarding other checkpoint molecules whose loss of function results in chemo-resistance. This is probably due to the fact that loss of function of these molecules occurs through very subtle genetic alterations which are not easily detected by any current molecular techniques. Research being performed under this award is focusing on the identification of genes whose loss of function results in the proliferation of malignant epithelial cells that are resistant to the cytotoxic action of taxol, a microtubule formation inhibitor that is used in the treatment of breast cancer. This is being performed with a novel system based on a recently described technique (8) that allows for the isolation of genes encoding selectable recessive phenotypes. Identifying molecular pathways that are altered in chemo-resistance tumor cells will be critical for the future design of novel therapeutic strategies restoring chemo-sensitivity to chemo-resistant breast tumor cells.

BODY

Overview of the in vitro knock-out system.

Described here is an in vitro knock-out approach that we have attepeted to use to try to identify genes that are important for mediating the cytotoxic action of specific chemotherapeutic drugs. The strategy is based on a technique described in the journal Cell by Dr. Stanley Cohen's laboratory that was used to identify cellular genes whose loss of function results in the transformation of immortalized mouse NIH3T3 cells (8). We have modified the previously used technique for two main reasons. First, we found that we could not transfect human epithelial cells with the retroviral gene search vector at a high enough frequency to insure the knock-out of a reasonable number of expressed cellular genes (using a number of packing cell lines, including the Bing, Bosc and Phoenix lines). Second, we believe that the use of an inducible promoter based system, instead of a constitutive one, will be helpfull to identify clones that have aquired drug resistance via the anti-sense mediated knock-out method and not via spontaneous genetic changes.

Figure 1 outlines our novel in vitro knock-out approach. An in vitro knock-out approach is being developed to identify genes that are important for mediating the cytotoxic action of specific chemotherapeutic drugs. The strategy employed (see figure 1) is a modified version of one that was used to identify cellular genes whose loss of function results in the transformation of immortalized NIH3T3 cells (8). This approach relies on a gene search insert and a cell line that contains high levels of the muristerone transcription factor, VgRXR. The gene search insert encodes a G418 resistance/B-galactosidase fusion protein. The fusion cDNA is downstream of a functional splice acceptor (SA) site, but is not downstream of a functional promoter. Therefore, expression of the fusion product is dependent on the integration of the gene search insert into transcribed sequences supplying a splice donor site. The first step in this process is to transfect the gene search insert into a target cell line containing high levels of the muristerone transcription factor, VgRXR. After transfection, cells are cultured in the presence of G418 to select for those cells harboring integration of the gene search insert into expressed cellular sequences. After two weeks of selection, those cells that are G418 resistant are pooled and expanded. Once a library of knock out cells have been generated, $1x10^7$ cells derived from at least 5,000 G418 resistant clones are then exposed to muristerone. Muristerone is an activator of the VgRXR transcription factor. Treatment of cells with this hormone will induce antisense transcripts complementary to sequences upstream of the gene search vector and in theory, should reduce the expression of protein encoded by the other allele. Twenty-four hours later, these muristerone treated cells will be exposed to a specific concentration of the chemotherapeutic drug taxol that kills close to 100% of the parental target cell line. Once drug resistant colonies have been isolated and expanded, the subsequent series of experiments will verify that the acquisition of drug resistance is mediated by the antisense transcript produced by the gene search vector, and not by spontaneous acquisition of drug resistance or by direct insertional inactivation by the transfected gene search construct. This will be performed by comparing the sensitivity of cells from the resistant colonies to drug and measuring the level of fusion protein -/= muristerone. Those cells that are resistant to the cytotoxic action of these drugs and possess low amounts of the fusion protein in the presence of muristerone, but not in its absence, will have likely acquired drug resistance through the antisense knock-out procedure. Standard recombinant DNA techniques will then be used to identify the specific cDNA whose loss of function results in the clonal proliferation of chemo-resistant cells. Finally, the isolated cDNA will be subcloned into a eukaryotic expression vector and transfected into chemo-resistant epithelial cells. Those cDNAs that reverse the drug resistance phenotype of the cells will be sequenced and characterized by data base searches.

Results from work performed in this first year of the award.

Generation of a potent inducible line: Work performed in the first year of this award focused on 1) generating a potent muristerone inducible line to insure that high levels of the antisense transcript

will be produced in each cell harboring the gene search insert. and 2) optimizing a transfection procedure such that a high number of G418 resistant colonies will be generated after transfection of the gene search insert. This is required to insure that a reasonable number of expressed cellular genes are being knocked-out. A muristerone inducible system (9) was chosen for our in vitro knock-out approach (see figure 2). The muristerone regulated transcription factor used in this system is a heterodymer derived from the drosophila ecdysone receptor (contains a modified VP16 transactivation domain) and the mammalian retinoid x receptor. Treatment of cells with the ecdysone analogue, muristerone, induces heteromeric complex formation of the two subunits, sequence specific binding of the complex to particular response elements (GRE's) and activation of downstream target genes. In order to generate an inducible line, human epithelial cells were transfected by the calcium-phosphate precipitation method with an expression construct (VgRXR, Invitrogen) encoding both subunits of the muristerone inducible transcription factor and a selectable marker (zeocin resistance). After transfection, cells were treated with 200 ug/ml zeocin for two weeks. Zeocin resistant colonies were isolated and expanded into cell lines. To test for inducibility, lines were transfected with a construct possessing a reporter gene (the B-galactosidase gene) located downstream of 5 hormonal responsive elements. After transfection, cells were split into two dishes. Twenty-four hours later, one of these dishes received muristerone (final concentration of 1mM). The concentration of muristerone used was the minimum concentration found to give the highest level of B-galactosidase induction by muristerone in parental cells transiently transfected with the VgRXR and B-galactosidase reporter constructs (pIND-Lacz) (data not shown). Extracts were prepared twenty-four hours later from treated and untreated dishes and the level of B-galactosidase activity determined. The amount of B-galactosidase activity in extracts from untreated cells were all found to be very similar (data not shown). However, the amount of B-galactosidase activity in extracts from muristerone treated cells differed dramatically between clones. Figure 3 shows the fold induction of reporter activity after muristerone treatment in the 14 different VgRXR transfected lines. Cells derived from clone 9 gave the highest fold induction of the 14 clones analyzed (approximately 10 fold).

The ability to reduce protein expression from the wild-type allele via muristerone induced antisense transcripts produced from the knock-out insert may be limiting in a line that gives only a 10 fold level of induction. We therefore set out to improve muristerone inducibilty in clone 9 cells. We decided to transfect these cells with the a VgRXR expression construct containing both a zeocin and puromycin selectable marker. This construct was generated by subcloning the puromycin resistance gene from pPuro (a Pvu II-BamH I fragment) into the BstE II site of VgRXR. This vector (VgRXR-puro) was then transfected into cells derived from clone 9 of the initial VgRXR transfectants and cells were cultured in the presence of zeocin (200 ug/ml) and puromycin (0.5 ug/ml) for two weeks. Zeocin/puromycin resistant colonies were isolated and expanded into cell lines. Testing for muristerone inducibilty was performed as described above using the same B-galactosidase reporter construct. Figure 4 shows the fold induction of reporter activity after muristerone treatment in 20 different VgRXR-puro transfected clone 9 cells. Cells derived from clone 8 gave the highest fold induction of the 14 clones analyzed (approximately 50 fold). To verify the fold induction in these cells (9-8 cells) using another reporter, cDNA encoding the mdm2 proto-oncogene was subcloned into the pIND vector. 9-8 cells were then transiently transfected with pIND and pIND-mdm2 and cells were processed as described above. Western blot analysis was then performed using extracts prepared from untreated and muristerone treated transfected cells. Figure 5 shows dramatically higher levels (at least 10 fold) of mdm2 protein in 9-8 cells transiently transfected with the muristerone inducible mdm2 construct (pIND-mdm2). Considering these cells possess endogenous levels of mdm2 and the calcium phosphate method can only transfect approximately 10% of these cells (data not shown), the total level of mdm2 inducibility in the transfected cell population is probably over 100 fold.

The level of muristerone induced B-galactosidase activity or the amount of mdm2 protein produced in 9-8 cells (between 50-100 fold) is close to that which is produced when either B-galactosidase or mdm2 is cloned downstream of a strong constitutive promoter (e.g. CMV promoter, SV40 promoter) (data not shown). These type of promoters have been used in the past

to produce antisense transcripts against target genes and block expression of proteins encoded by these genes (see references 10, 11 and 12 for examples). We therefore believe that these 9-8 cells should be able to produce enough antisense transcripts from the knock-out insert to significantly reduce the expression of protein encoded by the other wild-type allele.

Generation of a high number of G418 resistant colonies in gene search insert transfected cells: Besides the generation of an inducible cell line, the second area where substantial progress was made during the first year of this award was the generation of a knock-out cell library. An acceptable knock-out library will depend on the efficient transfection of the 9-8 cells with the gene search vector and the generation of a large number of G418 resistant cells. We performed many small scale transfection experiments to optimize the generation of G418 resistant colonies. For example, comparisons between transfection procedures (e.g. electroporation vs lipofectamine vs calcium phosphate), percent confluency prior to transfection, amount of DNA used in the transfection and concentrations of G418 used in selection were all performed to determine the best method for generating the highest number of G418 resistant clones. A method was developed from all of these studies that was able to generate approximately 130 G418 resistant colonies per transfection point (2×10^7 cells). After scaling up to 50 transfection points, we have been able to generate a knock-library of approximately 6,500 G418 resistant colonies. To verify insertion of the G418 resistant/B-galactosidase fusion sequence from the gene search insert into expressed cellular sequences, northern blot analysis on RNA isolated from some of these colonies was performed. Using a probe to the G418 resistant/B-galactosidase fusion sequences, we were able to detect transcripts that were larger than the predicted size of the G418 resistant/B-galactosidase fusion sequences alone (figure 5). Northern blot analysis has been performed using RNA from 5 more lines (data not shown) and the average number of fusion transcripts detected in all lines analyzed is approximately three. These results suggest that integration of the gene search vector has occurred downstream of expressed cellular sequences in the 9-8 cells. Considering that our library is derived from 6,500 G418 resistant clones and each of these clones has at least 3 integration events, we estimate that our cell library contains the disruption of approximately 20,000 expressed cellular genes in 9-8 cells. We do realize that this estimate is based on some assumptions (i.e. transcripts detected are from different genes-not splice variants from the same gene, our northern blot analysis can detect all transcripts derived from integration of the gene search insert into expressed cellular sequences). Nonetheless, because it has been estimated that there are 100,000 genes and 10% of these are expressed in any given cell type, it appears that we have been able to disrupt a reasonable percentage of genes in 9-8 cells. Work described in year 1 meets technical objectives (#1-#3) proposed in the statement of work outlined in the initial grant application.

Summary of work performed in the second year of the award.

Optimization of drug survival assays and the selection of drug resistant cells harboring the gene search and transactivating vectors (technical objective #4): Cells derived from the knock-out library were seeded into 150 x 25 mm dishes and cultured in the presence of zeocin (100 ug/ml), G418 (200 ug/ml) and puromycin (0.5 ug/ml). When cells reached approximately 90% confluency, they were treated with varying concentrations of taxol for 2 hours. After treatment, cells were washed in phosphate-buffered saline (PBS) and re-fed with complete media containing the same concentrations of zeocin, G418 and puromycin. Cells were washed and re-fed every other day for two weeks. After two weeks, the number of taxol resistant colonies were counted (see Table 1). Based on the numbers obtained, we chose the minimal concentration of taxol used (1 ug/ml) that gave a minimum background of resistant colonies to try to identify those cells in the knock-out library that are resistant to the cytotoxic action of this drug in the presence of the functional transactivator.

To identify these cells, cells derived from the knock-out library were seeded into 10 100 x 15 mm dishes and cultured in the presence of zeocin, G418 and puromycin. When cells reached

approximately 50% confluency, they were transfected by the calcium phosphate methods with the pVgRXR expression. This was performed to insure maximal expression of the muristerone inducible transactivator. After transfection, cells were seed into 10 150 X 25 mm dishes and cultured in the presence of zeocin, G418 and puromycin for one day. Cells were then pre-treated for 2 hours with 20 uM muristerone, then treated with 1ug/ml of taxol for two-hours in the presence of 20 uM muristerone. Muristerone is used as a hormone to activate the transactivator and increase the levels of antisense RNA that is complementary to sequences where the gene search insert the has integrated. This should reduce the expression of protein encoded by both alleles of the gene containing the B-galactosidase/G418 fusion. After taxol treatment, cells were washed in PBS, re-fed with complete media containing muristerone, zeocin, G418 and puromycin and incubated overnight at 37°C. After the overnight incubation, cells were washed and re-fed with media containing only zeocin, G418 and puromycin. Cells were washed and re-fed with this media every other day for two weeks. After two weeks, 20 colonies were identified to be resistant to taxol in muristerone co-treated cells.

To identify transactivator mediated chemo-resistant tumor cells (i.e. those that are more resistant to taxol in the presence of muristerone than in its absence) (technical objective #5): After completion of technical objective #4, the next goal was to identify those colonies whose resistance to taxol is dependent on the presence of the functional transactivator (i.e. more resistant to taxol in the presence of muristerone than in its absence). Individual colonies were first isolated using cloning cylinders and trypsin and seeded into T75 flasks. Cells were cultured in the presence of zeocin, G418 and puromycin until they reached 50% confluency (this took approximately 1 month). After cells reached 50% confluency, cells were trypsinized and viable frozen cells were prepared from half of the cells while the other half was re-seeded into 100 X 15 mm dishes. Cells were again transfected with the pVgRXR expression construct by the calcium phosphate method. After transfection, cells were split into two dishes. The next day, one of these dishes was treated with muristerone and taxol as described above. The other dish was treated in a similar manner, except that muristerone was not included in the media. Two weeks later, the overall viability of cells were determined in all of the 20 clones -/+ muristerone treatment. The majority of clones were very sensitive to taxol, regardless of whether they were treated with muristerone (see Table 2). There were two clones that were relatively resistant to taxol (clones 12 and 16), but again there were no difference in the sensitivity of these clones -/+ muristerone treatment. We were able to identify two clones (clones 13 and 17) that were much more resistant to the action of taxol in the presence of muristerone than in its absence. These clones may contain an antisense mediated knock-out of a protein that is important in mediating sensitivity to taxol. In the absence of muristerone, this protein is presumably expressed at high enough levels to block tumor cell proliferation in the presence of taxol. However, when the expression of this protein is blocked via muristerone-induced antisense RNA production, the cells are relatively resistant to this chemotherapeutic drug.

Summary of work performed in the third year of the award.

Muristerone does not reduce the expression of the B-galactosidase fusion product: Prior to proceeding to work outlined in technical objectives #6 and #7, we first wanted to make sure that muristerone-induced antisense production leads to a decrease in the G418 resistant/B-galactosidase fusion proteins that are present in the taxol resistant lines. If muristerone treatment leads to a decrease in fusion message, this result would significantly increase our confidence that taxol resistance is due to anti-sense mediated knock-out of gene expression. Clones derived from lines 13 and 17 were again transfected with the VgRXR expression construct as described above (this was performed to maximize antisense production). Because we wanted to analyze fusion protein levels in the transfected cell population, all transfection included the pHOOK-1 plasmid (Invitrogen). After transfection, cells were split into 100 mM dishes and treated with media alone or with media containing muristerone for twenty four hours. Cells were then treated with either taxol alone or taxol and muristerone for 2 hours. Cells were then washed and re-fed with media

alone or with media containing muristerone for another twenty-four hours. After this incubation, cells were harvested and transfected cells were isolated using Capture-Tec beads according to the manufacturer's instructions (Invitrogen). Extracts were then prepared and a western blot was performed with an anti B-galactosidase antibody. As depicted in Figure 7, we found no difference in the level of fusion proteins -/+ muristerone treatment. Specifically, line #17 possessed a single in-frame integration event and there was no difference in the level of fusion protein in muristerone treated and in un-treated cells. Line #13 possessed two in-frame integration events and again, there was no evidence for down-regulation of either of these proteins in the muristerone treated cells. We not only repeated this same experiment a number of times, but we also performed experiments where we used different concentrations of muristerone, varied the time of incubation with muristerone, and changed the concentration of taxol. We also performed these experiments in other lines that possessed lower levels of fusion proteins (and thus may be more sensitive to antisense mediated down-regulation). Unfortunately, we did not find any indication that the levels of fusion protein were lower in muristerone treated cells when compared to un-treated cells. These results were highly discouraging because it suggests that taxol resistance is due to random genetic changes that have occurred in these cells and is not due to anti-sense mediated knock-generated by the gene fusion insert. Because of these results, we did not initiate work to determine the identity of the genes where the gene search insert had integrated.

RESEARCH ACCOMPLISHMENTS

1) Generation of an inducible cell line.

2) Identification of two new mechanims by which the expression of the MDM2 onco-protein can be de-regulated in human tumor cells (see CONCLUSIONS AND SO WHAT section for more information relating to this area).

REPORTABLE OUTCOMES

Manuscripts/Abstracts:

Pan, Y., and D.S. Haines. The pathway regulating MDM2 protein degradation can be altered in human leukemic cells. Cancer Research 59:2064-2067, 1999.

Pan, Y. and D.S. Haines. Identification of a tumor-derived p53 mutant with novel transactivating selectivity. Oncogene 19:3095-3100, 2000.

Degrees obtained that are supported by this award:

Yi Pan earned a Ph.D. in Molecular Biology at Temple University and is now performing a post-doctoral fellowship at the University of Pennsylvania.

Development of cell lines:

We have generated a very good inducible cell line that we will make available to other investigators upon request after we have published a manuscript that describes these items.

Funding applied for based on work supported by this award:

American Cancer Society (4/3/2000), "p53 mutants that up-regulate MDM2", Dale S. Haines, Ph.D., P.I.

National Institutes of Health (6/1/2000), MDM2 expression in p53 mutant cells, Dale S. Haines, Ph.D., P.I.

Employment opportunities:

The P.I.was awarded a grant to pursue these studies while working as an Assistant Professor at Allegheny University of the Health Sciences. After the declaration of bankruptcy at Allegheny, it became very difficult to perform research at this Institution. Because of the amount of funding that had been obtained by the P.I. (including for this project), the P.I. was able to move his laboratory to a more respected Institution that possesses far better resources. Our move to the Fels Institute for Cancer Research and Molecular Biology at Temple University would probably not have been possible without the support of the US army and should enable the P.I. to become a more productive tumor biologist in the future.

CONCLUSIONS

We have attempted to establish a random in vitro knock-out approach to identify genes that mediate chemosensitivity to taxol in human mammary epithelial cells. Unfortunately, we found that this system has several limitations, including the inability to block expression of transcripts that encode for the protein where the gene search insert has integrated. The inability to block expression makes it very likely that cells derived from lines 13 and 17 have acquired drug resistance via non-specific effects of muristerone and random genetic changes that have occurred in the cells upon exposure to these compounds. Because we did not see any evidence for antisense-mediated reduction, we feel that this approach will only work if loss of heterozygous is sufficient for the emergence of a drug resistant phenotype. We are currently comparing taxol sensitivity in lines 13 and 17 to other lines to determine if there is a statistically significant difference in sensitivity between these lines. If so, we will then determine the identity of the endogenous cDNAs in the B-galactosidase/G418 fusions and if any of the cDNAs can restore sensitivity when overexpressed in these cells.

Because of the high risk nature of the proposed work and the fact that a student's thesis was dependent on studies supported by this award, we also pursued some interesting findings that were not directly related to the statement of work that were observed during the course of these studies. The subject of these findings are presented in the enclosed manuscripts. These published studies, as well as the awarding of a doctoral degree to Yi Pan from Temple University, would not have been possible without this award and we thank the Department of Defense for supporting all of the studies described in this final report.

REFERENCES

1. Lowe, S.W., S. Bodis, A. McClatchey, L. Remington, H.E. Ruley, D.E. Fisher, D.E. Housman, and T. Jacks. 1994. p53 status and the efficacy of cancer therapy in vivo. Science 266:807-810.
2. Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 193. p53 dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 80:957-967.
3. Wattel E, C. Preudhomme, B. Hecquet , M. Vanrumbeke, B. Quesnel, I. Dervite, P. Morel, and P. Fenaux. 1994. p53 mutations are associated with resistance to chemotherapy and short survival in hematological malignancies. Blood 84:3148-3157.
4. Martin H.M, M.I. Filipe, R.W. Morris, D.P. Lane, and F. Silvestre. 1992. p53 expression and prognosis in gastric carcinoma. Int J Cancer 50:859-862.
5. Horio Y, T. Takahashi , K. Hibi, M. Suyama, T. Niimi, K. Yamakawa, Y. Nakamura, R. Ueda, and T. Takahashi. 1993. Prognostic significance of p53 mutations and 3p deletions in primary resected non-small cell lung cancer. Cancer Research 53:1-4.
6. Thor A.D, D.H. Moore, S.M. Edgerton, E.S. Edgerton, E.S. Kawassaki, E. Reihsaua, H.T. lynch, J.N. Marcus, L. Schwartz, L.C. Chen, B.H. Mayall, and H.S. Smith. 1992. Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancer. J Natl Cancer Inst 84:845-855.
7. Wosikowski, K., J.T. Regis, R.W. Robey, M. Alvarez, J.T. Buters, J.M. Gudas, and S.E. Bates. 1995. Normal p53 status and function despite the development of drug resistance in human breast cancer cells. Cell Growth and Differentiation 6:1395-13403.
8. Li, L. and S. Cohen. 1996. tsg101:A novel tumor suppressor gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. Cell 85:319-329.
9. No, D., T.-P. Yao and R.M. Evans. 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. sci. USA 93:3346-3351.
10. Izem, L., E. Rassart, L. Kamate, L. Falstrault, D. Rhainds and Brissette. 1998. Effect of reduced low-density lipoprotein receptor level on HepG2 cell cholesterol metabolism. Biochemical Journal 329:81-89.
11. Hiraiwa N., Dohi T., Kawakami-Kimura N., Yumen M., Ohmori K., Maeda M. and R. Kannagi. 1996. Suppression of sialyl Lewis X expression and E-selectin-mediated cell adhesion in cultured human lymphoid cells by transfection of antisense cDNA of an alphal 3-fucosyltransferase. J. Biol. Chem. 271:31556-31561.
12. Rabbani, S.A., J. Gladu, B. Liu and D. Goltzman. 1995. Regulation in vivo of the growth of Leydig cell tumors by antisense ribonucleic acid for parathyroid hormone-related peptide. Endocrinology 136:5416-5422.

FINAL REPORT

Bibliography:

Publications:

Pan, Y., and D.S. Haines. The pathway regulating MDM2 protein degradation can be altered in human leukemic cells. Cancer Research 59:2064-2067, 1999.
Pan, Y. and D.S. Haines. Identification of a tumor-derived p53 mutant with novel transactivating selectivity. Oncogene 19:3095-3100, 2000.

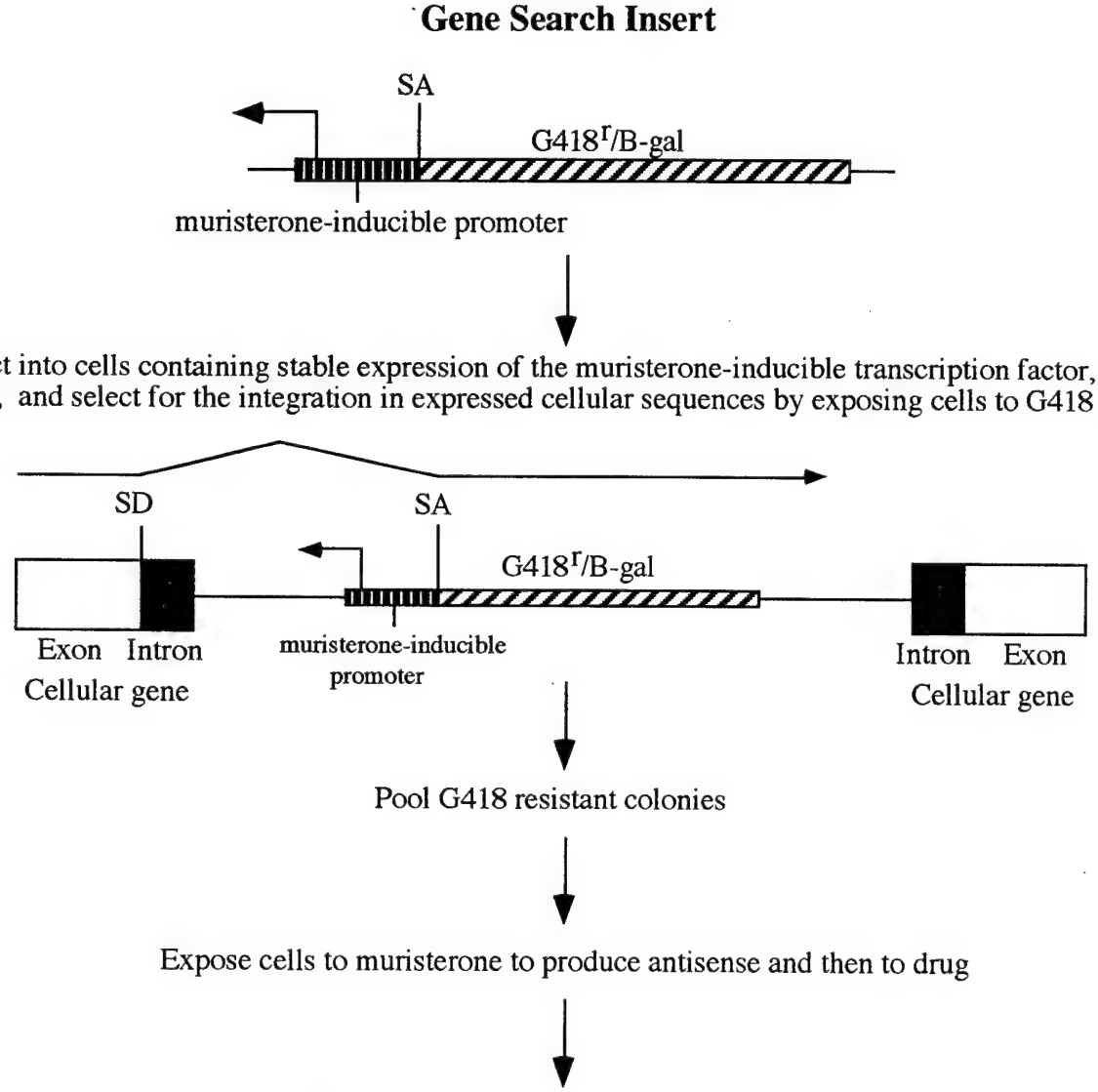
Meeting abstracts:

Pan, Y., and D.S. Haines. The pathway regulating MDM2 protein degradation can be altered in human leukemic cells. AACR annual meeting, Philadelphia PA, 1999.
Pan, Y. and D.S. Haines. Identification of a tumor-derived p53 mutant with novel transactivating selectivity. DOD Era of Hope meeting, Atlanta GA, 2000.

List of Personnel receiving pay from the research effort at Temple University:

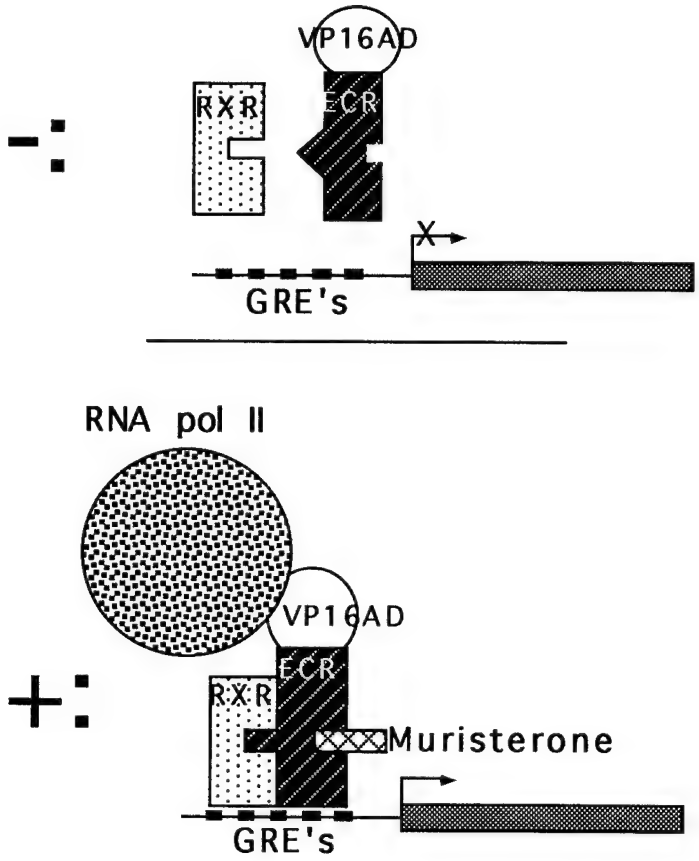
Yi Pan, Ph.D.
Dale S. Haines, Ph.D.

Figure 1. Generation of an in vitro cell knock-out library



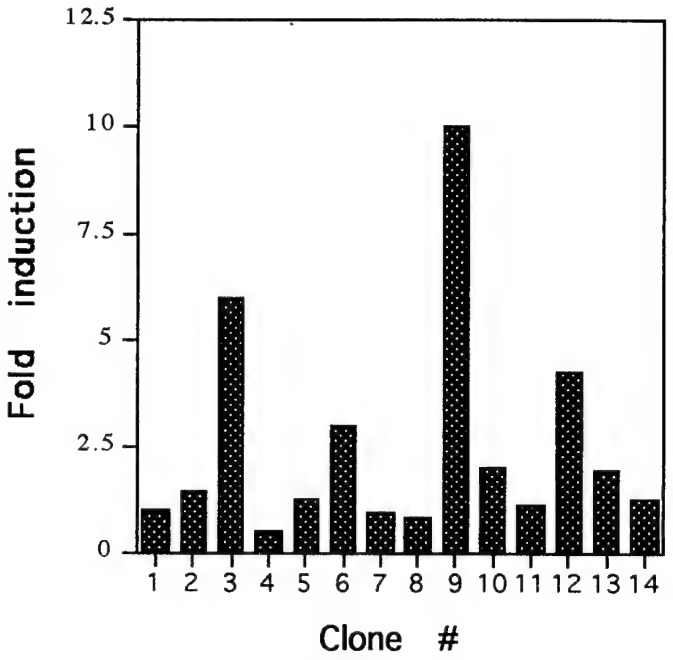
Legend. Method for using a random knock-out procedure to identify genes that mediate the cytotoxic action of chemotherapeutic drugs.

Figure 2. The muristerone inducible transcriptional activation system



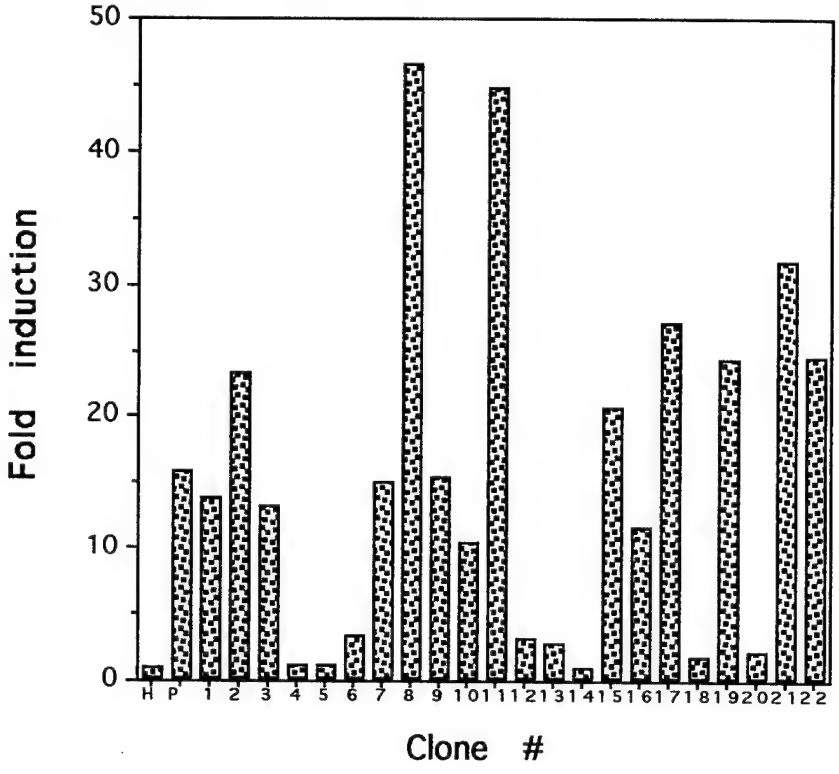
Legend. Schematic representation of the mechanism by which the expression of genes downstream of the muristerone inducible promoter are regulated in the absence (-) or presence (+) of this hormone.

Figure 3. Fold induction of B-galactosidase activity in muristerone treated VgRXR transfected lines



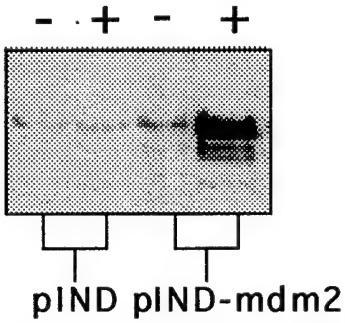
Legend. Zeocin resistant, VgRXR transfected lines were transfection with a construct containing the B-galactosidase reporter gene under the control of 5 hormonal responsive elements. Extracts were prepared from muristerone treated and untreated cells and B-galactose activity measured. Fold induction was calculatedas follows: $\frac{\text{B-gal. activity(+ murs.)}}{\text{B-gal. activity(- murs.)}}$

Figure 4. Fold induction of B-galactosidase activity in muristerone treated VgRXR-puro transfected lines



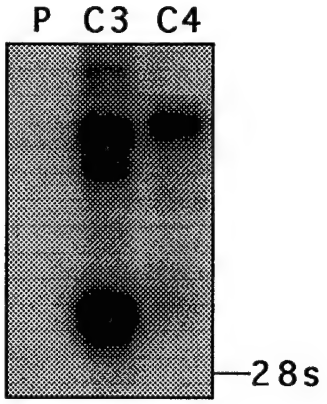
Legend. Zeocin/puromycin resistant lines were transfection with a construct containing the B-galactosidase reporter gene under the control of 5 hormonal responsive elements. Extracts were prepared from muristerone treated and untreated cells and B-galactosidase activity measured. Fold induction was calculated as follows: $\frac{\text{B-gal. activity(+ murs.)}}{\text{B-gal. activity(- murs.)}}$

Figure 5. Induction of mdm2 protein in muristerone treated 9-8 cells transfected with pIND-mdm2



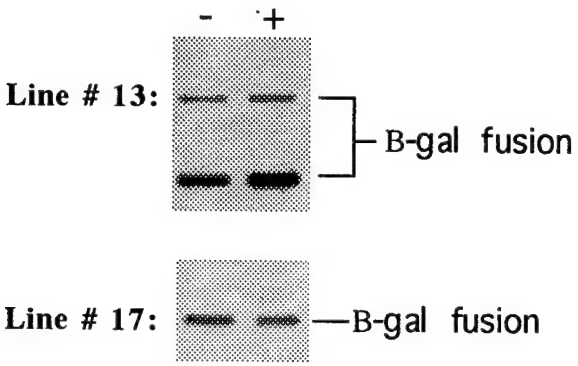
Legend. 9-8 cells were transfected with pIND or pIND-mdm2 by the calcium phosphate precipitation method. Twenty four hours after transfection, cells were split into dishes. One of these dishes was treated with muristerone (+) while the other one was not. Extracts were prepared 24 hours later and the amount of mdm2 protein measured by a western blot using the mdm2 Ab-1 monoclonal antibody (Calbiochem).

Figure 6. Northern analysis of clones isolated from the 9-8 cell knock-out library.



Legend. A northern blot containing RNA from parental 9-8 cells (P) and different G418 resistant colonies (C3 and C4) isolated from the 9-8 knock-out library was probed with a G418 resistance/B-galactosidase fusion DNA probe. After hybridization, blot was washed in 0.2X SSC/0.1% SDS at 65°C and exposed to x-ray film.

Figure 7. Muristerone does not reduce the expression of the B-galactosidase fusion proteins in the taxol resistant lines #12 and #17.



Legend. Lines #13 and #17 were seeded into 100 mM dishes and transfected with the VgRXR expression construct and the pHOOK plasmid by the calcium phosphate precipitation method. After transfections, cells were split into 100 mM dishes and treated with media alone (-) or with media containing 3 uM muristerone (+) for twenty four hours. Cells were then treated with either 1 ug taxol (-) or taxol and muristerone (+) for 2 hours. Cells were then washed and re-fed with media alone (-) or with media containing 3 uM muristerone (+) for another twenty four hours. Cells were harvested and transfected cells were isolated using Capture-Tec beads according to the manufacturer's instructions (Invitrogen). Extracts were then prepared and a western blot was performed with an anti B-galactosidase antibody.

Table 1. Sensitivity of cells from the knock-out library to taxol

Taxol Concentration (ug/ml)	# colonies
30	2
10	1
3.0	2
1.0	3
0.3	>100

Legend. 9-8 cells were treated with varying concentrations of taxol for 2 hours. After treatment, cells were washed and re-fed every other day with media containing zeocin, G418 and puromycin. The number of colonies were then determined two weeks later.

Table 2. Sensitivity of cells derived from the 20 independent clones to taxol -/+ muristerone treatment.

	# of colonies two weeks after taxol exposure	
Clone #	**+ muristerone**	**- muristerone**
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	>500	> 500
13	350	50
14	1	5
15	0	0
16	>500	>500
17	130	22
18	0	0
19	0	0
20	0	0

Legend. Taxol resistant clones were transfected with the pVgRXR expression plasmid and seeded into two dishes as described in the Body Section of this report. One of these dishes was treated with taxol and muristerone and one was treated with taxol alone. Two weeks later, the number of taxol resistant colonies were determined.

Oncogene (2000) 19, 3095–3100

www.nature.com/onc

SHORT REPORT

Identification of a tumor-derived p53 mutant with novel transactivating selectivity

Yi Pan[1] and Dale S Haines*,[1]

[1]The Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, 3307 N. Broad Street, Philadelphia, Pennsylvania, PA 19140, USA

MDM2 is a p53-responsive molecule that when overexpressed, can alter growth control pathways via p53-dependent and independent mechanisms. We have identified a mutant p53 containing line that expresses high levels of transcripts that are regulated by the p53-responsive promoter of the *MDM2* gene. Analysis of cloned product obtained from these tumor cells revealed that they harbor a mutant p53 protein (possessing an Arg to Gln substitution at codon 213) that is a potent transactivator of MDM2 expression. Consistent with this activity, the R213Q mutant was found to have the ability to interact with DNA sequences located within the MDM2 promoter. In contrast to previously described tumor-derived p53 mutants which retain MDM2 transactivation function and possess partial growth suppressive activity, the R213Q mutant is severely compromised in its ability to induce p53-regulated transcripts that encode for proteins involved in cell-cycle arrest and apoptosis. The R213Q mutant can also be expressed at high levels in stably transfected cells and cells that harbor this mutant possess elevated levels of MDM2 protein. The R213Q mutant was also found to be able to up-regulate MDM2 during a genotoxic stress response. R213Q is the first described tumor-derived p53 mutant that is deficient at up-regulating both cell cycle arrest and apoptotic factors, but is highly proficient at inducing the growth-promoting molecule MDM2. *Oncogene* (2000) **19**, 3095–3100.

Keywords: p53; MDM2; p53 target genes

The functional activity of the p53 tumor suppressor protein is inactivated by mutation in a large percentage of human tumors (Hainaut *et al.*, 1998). It is highly probable that the most important biochemical activity of p53 is to function as a transcription factor. p53 is capable of binding to DNA in a sequence specific manner and influencing gene expression (el-Deiry *et al.*, 1992; Funk *et al.*, 1992; Zambetti *et al.*, 1992). The majority of tumor derived p53 mutants harbor amino acid changes in the DNA binding domain (Cho *et al.*, 1994) and many mutants are unable to bind to specific promoter elements (el-Deiry *et al.*, 1992; Funk *et al.*, 1992). Thus, mutational inactivation of p53 binding function for sequences that are recognized by wild-type (wt) p53 is likely to be a key mechanism which contributes to the development of a tumor cell.

It is now becoming apparent that mutations in the *p53* gene do not necessarily give rise to an inert protein. Considering that p53 functions as a tetramer (Wang *et al.*, 1995), it is not surprising that p53 mutant proteins can block the transcriptional activity of wt p53 (Kern *et al.*, 1992). Tumor-derived p53 mutants have also been identified that are able to induce cell cycle arrest, but not apoptosis (Friedlander *et al.*, 1996; Rowan *et al.*, 1996), and this appears to be associated with the inability of these mutants to bind to a subset of p53-response elements (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996). Interestingly, some tumor-derived p53 mutants which have lost wt p53 function appear to have acquired the ability to provide a selective growth advantage to the tumor cell (Roemer, 1999). This 'gain of function' activity is thought to be due to their ability to up-regulate the expression of growth promoting molecules that are not targets of wt p53. In support of this theory, the promoter elements of *myc* (Frazier *et al.*, 1998), *BAG-1* (Yang *et al.*, 1999) and the *multidrug resistant* genes (Lin *et al.*, 1995) have been shown to be mutant p53-responsive.

The *MDM2* gene is a transcriptional target of wt p53 (Barak *et al.*, 1993; Wu *et al.*, 1993). The protein product of the *MDM2* gene is a natural negative regulator of p53 activity (Montes de Oca Luna *et al.*, 1995; Jones *et al.*, 1995) and is oncogenic when overexpressed (Fakharzadeh *et al.*, 1991). The functional properties of MDM2 are therefore unique when compared to other transcriptional targets of p53, since it is involved in the promotion and not the inhibition of cellular proliferation. It is generally accepted that p53 function is compromised in MDM2 overexpressing cells, and there is now good evidence which suggests that the activity of multiple growth control molecules may be de-regulated in addition to p53 in MDM2 overproducing tumors. MDM2 has been shown to interact with a number of growth control molecules (Xiao *et al.*, 1995; Martin *et al.*, 1995). Moreover, MDM2 overexpression can promote cell growth via p53-independent mechanisms (Dubs-Poterszman *et al.*, 1995; Sigalas *et al.*, 1996; Sun *et al.*, 1998; Lundgren *et al.*, 1997; Jones *et al.*, 1998) and p53 mutations have been detected in MDM2 overexpressing tumors (Marks *et al.*, 1996; Pan and Haines, 1999; Eischen *et al.*, 1999). This genetic analysis, in combination with the MDM2 functional studies described above, are good indicators that MDM2 overexpression can provide a growth advantage to a p53 mutant tumor cell.

*Correspondence: DS Haines
Received 19 April 2000; revised 27 April 2000; accepted 4 May 2000

The MDM2–P2 promoter possesses two p53-response elements and it controls the expression of transcripts (termed MDM2–P2 transcripts) in a p53-dependent manner (Wu *et al.*, 1993; Zauberman *et al.*, 1995). In a previous study, we demonstrated that the levels of MDM2–P2 transcripts can be used to predict *p53* gene status in human leukemic cell lines (Bull *et al.*, 1998). In a later survey of more human leukemic cell lines, we noticed one mutant p53 containing line (Duthu *et al.*, 1992) that expresses elevated levels of MDM2–P2 RNA. Figure 1 shows that the level of MDM2–P2 transcripts present in the Raji cell line (R) is much higher than that measured in other p53 mutant lines. These results raise the possibility that p53-independent mechanisms are responsible for the production of MDM2–P2 transcripts in Raji cells. Alternatively, this cell line may harbor a p53 mutant that is proficient at up-regulating *MDM2* gene expression. To investigate this possibility, full-length p53 cDNAs were cloned from this line and their sequence determined. Sequence analysis revealed that the Raji line harbors two different mutant alleles: one of these alleles encodes for a mutant protein (the R213Q mutant) that possesses an Arg to Gln amino acid change at codon 213, while the other allele encodes for a mutant protein (the Y234H mutant) that contains a Tyr to His substitution at codon 234 of p53. These mutations are identical to those previously reported to be present in the Raji line (Duthu *et al.*, 1992). To determine if one or both of these mutants possesses the ability to up-regulate MDM2 expression, the R213Q and Y234H mutants were cloned into a eukaryotic expression vector (pCEP, Invitrogen) and transfected into the p53 null H1299 cell line. Protein extracts were prepared from transiently transfected cells and the level of MDM2 protein was measured by a Western blot. Figure 2a shows that the R213Q mutant is able to up-regulate MDM2 protein in transiently transfected H1299 cells. No apparent induction of MDM2 protein was observed in cells that had been transfected with the Y234H mutant. Surprisingly, the fold induction observed with the R213Q mutant was similar to that measured with a pCEP wt p53 expression construct at all amounts of input DNA tested. These results strongly suggest that MDM2–P2 production in Raji cells is due to the presence of a p53 mutant that is able to up-regulate the expression of these transcripts.

We next wanted to determine if the R213Q mutant can bind to DNA sequences located within the MDM2–P2 promoter. Assessment of binding to the

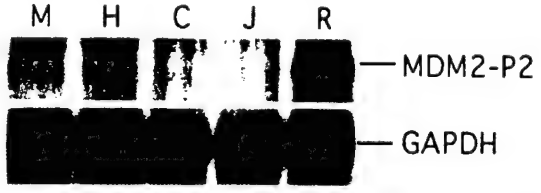


Figure 1 MDM2-P2 RNA levels are elevated in the p53 mutant Raji cell line. Measurement of MDM2-P2 transcript was performed by RNase protection as described (Bull *et al.*, 1998) with cellular RNA isolated from the p53 mutant lines Molt4 (M), HEL (H), CEM (C), Jurkat (J), and Raji (R). All hybridization reactions included a smaller probe complementary to the glyceraldehyde-phosphate-dehydrogenase (GAPDH) 'housekeeping' gene to control for potential variability in sample processing

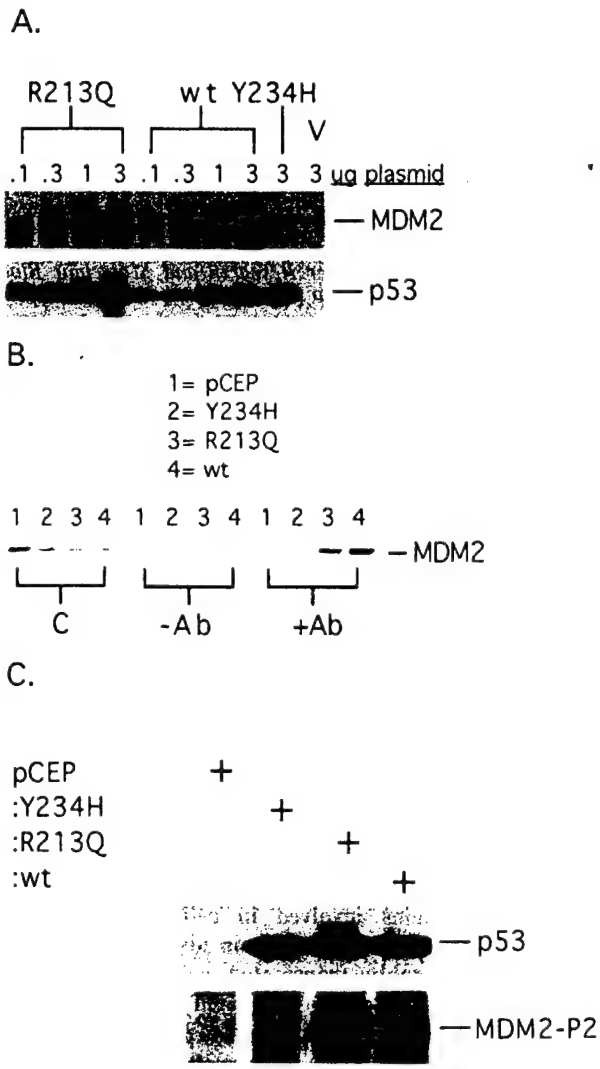


Figure 2 R213Q retains MDM2 transactivation function. (**a**) H1299 cells were transfected with varying amounts of wt p53 and the R213Q mutant and 3 μg of the Y234H mutant and the pCEP Vector as described previously (Haines *et al.*, 1994) (V). Forty-eight hours after transfection, protein extract was prepared and the amount of MDM2 and p53 protein was measured by a Western blot as described previously (Pan and Haines, 1999). Ponceau S. staining of blots after transfer revealed equivalent loading of total protein (data not shown). (**b**) Cells were transfected with the indicated plasmids and ChIP was performed as described previously (Murphy *et al.*, 1999). PCR was performed using primers complementary to sequences (available upon request) flanking the p53 response elements located within the MDM2 promoter and DNA (at two different dilutions) isolated after immunoprecipitation with the rabbit p53 polyclonal (+Ab) or the beads alone as control (−Ab). 'C' denotes PCR reactions using the total chromatin control. PCR products were separated on 1.5% agarose gels and stained with Ethidium Bromide. Presented here are pictures of PCR products derived from non-diluted DNA (in the case of the immunoprecipitations) or the least diluted DNA (in the case of the total chromatin controls). Reactions containing the least amount of DNA either gave no signal (for all control immunoprecipitations and for p53 immunoprecipitations from pCEP and pY234H transfected cells) or signal that was too faint to see after the scanning of pictures (for the total chromatin control reactions and for p53 immunoprecipitations from wt p53 and R213Q transfected cells). Two different dilutions were used to verify that PCR was performed under conditions that did not exceed the linear range of amplification. (**c**) H1299 cells were transfected with 5 μg each of vector (pCEP), the Y234H mutant, the R213Q mutant and wt p53. Protein and RNA was prepared 48 h after transfection. The levels of p53 protein in transfected cells was measured by a Western blot and the levels of MDM2-P2 transcripts by RNase protection

endogenous MDM2 promoter by transfected R213Q was assessed by a chromatin immunoprecipitation assay (ChIP) (Boyd *et al.*, 1998). This approach was chosen over traditional *in vitro* DNA binding assays because it allows for the study of protein–promoter interactions in the context of the endogenous genomic environment. wt p53, the R213Q mutant and a MDM2 transactivating deficient mutant control (Y234H) were again transfected into H1299 cells. DNA–protein interactions were first stabilized in transfected cells by chemical cross-linking. After preparation of nuclear extracts and DNA sonication, p53-DNA complexes were immunopurified using polyclonal p53 antibodies and protein A/DNA sepharose. DNA was further purified and PCR using this DNA and primers complementary to sequences flanking the p53-binding site of the MDM2 promoter was performed. Figure 2b shows that there was a greater amount of PCR product present in samples that were derived from p53 immunoprecipitates of R213Q and wt p53 transfected cells when compared to the control samples (i.e. samples derived from p53 immunoprecipitates of vector control and R234H transfected cells and in the control immunoprecipitations ('–Ab' lanes) from all of the transfections). Since equivalent amount of product was observed in PCR reactions using DNA that was prepared from a fraction of the extract prior to immunoprecipitation ('C' lanes), it is unlikely that the signal observed in the R213Q or wt p53 samples is simply due to a higher amount of input DNA. The presence of signals in samples derived from wt p53 and R213Q transfections does not appear to be due to a general higher affinity of these proteins for DNA since no PCR product was observed in PCR reactions using primers complementary to a control sequence (i.e. the coding region of the TGF-B type II receptor, data not shown). These results suggest that the R213Q mutant can bind to DNA sequences that are located within the MDM2–P2 promoter. Consistent with the binding data, elevated amounts of MDM2–P2 transcripts were present in R213Q transfected cells (see Figure 2C). These results strongly suggest that R213Q is able to induce MDM2 protein expression by binding to sequences located within the MDM2–P2 promoter and up-regulating the expression of transcripts that are under the control of this promoter.

The results presented above show that the R213Q mutant retains transactivation function on the MDM2–P2 promoter. To determine if the R213Q mutant possesses the ability to up-regulate growth suppressive molecules that are induced by wt p53, we next measured the levels of wt p53-responsive transcripts p21 (el-Deiry *et al.*, 1993), BTG-2 (Rouault *et al.*, 1996), PIG3 (Polyak *et al.*, 1997) and PIG11 (Polyak *et al.*, 1997) in transfected cells. These were chosen because like MDM2, they are very strongly induced by wt p53 (Zhu *et al.*, 1998) and they encode for molecules that have been implicated in distinct p53-controlled responses. p21 and BTG-2 are known to play important roles in controlling cell cycle progression (Montagnoli *et al.*, 1996; Rouault *et al.*, 1996; Waldman *et al.*, 1995). In contrast, PIG3 and PIG11 have been postulated to participate in p53-induced apoptosis (Polyak *et al.*, 1997). We also included two other p53 mutants (R175P and R181L) in this analysis (these were generated by site-directed mutagenesis using pCEP-wt p53 as the template). These mutants possess the ability to up-regulate MDM2 and p21, and can induce cell cycle arrest (Crook *et al.*, 1994; Ludwig *et al.*, 1996; Rowan *et al.*, 1996). They are, however, defective in their ability to induce apoptosis (Ludwig *et al.*, 1996; Rowan *et al.*, 1996). RNA was isolated following transfection and Northern analysis for p21, BTG-2, PIG3 and PIG11 was performed. Figure 3a shows high levels of MDM2 protein in cells that had been transfected with the R213Q, R175P and R181L mutants. However, in contrast to cells transfected with the R213Q mutant, cells transfected with the R175P and R181L mutants express much higher levels of p21, PIG3, PIG11 and BTG-2 RNA (Figure 3b). These results suggest that the R213Q mutant is defective at inducing the expression of putative growth suppressive proteins but is proficient at inducing MDM2 protein expression. They also indicate that the R213Q mutant

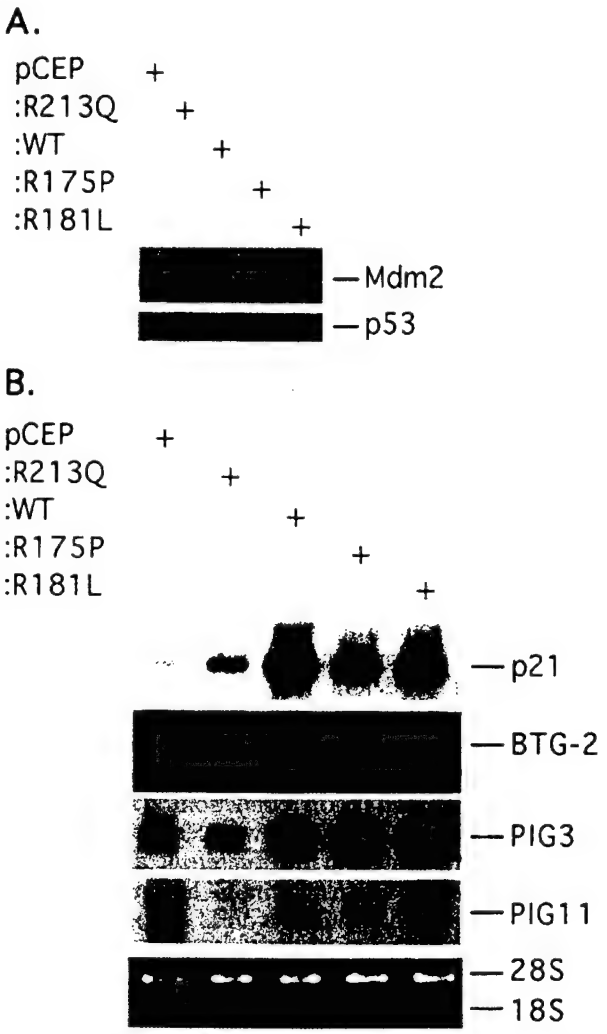


Figure 3 Induction of p53-responsive genes by the R213Q, R175P and R181L p53 mutants. H1299 cells were transfected with 5 μg each of vector (pCEP), the R213Q mutant, wt p53, the R175P mutant, and the R181L mutant. Cells were harvested 48 h after transfection for protein and RNA preparation. (**a**) p53 and MDM2 protein levels were measured by a Western blot. Equivalent blotting of protein for this Western blot was verified by Ponceau S. staining (data not shown). (**b**) The levels of p21, BTG-2, PIG3 and PIG11 in transfected cells was determined by Northern analysis. Also depicted is the ethidium stain of the agarose gel prior to transfer to show equivalent loading of RNA

is unique when compared to previously described mutants that retain MDM2 transactivation function.

Because MDM2 can promote cell growth via p53-independent mechanisms when overexpressed *in vitro* (Dubs-Poterszman *et al.*, 1995; Sigalas *et al.*, 1996; Sun *et al.*, 1998) and *in vivo* (Lundgren *et al.*, 1997; Jones *et al.*, 1998), we next wanted to determine if MDM2 protein could be further up-regulated by this mutant in cells exposed to a genotoxic stress. A pool of clones derived from R213Q stably transfected H1299 cells were treated with the DNA cross-linking agent cisplatin. The levels of MDM2 protein were then measured in treated and untreated cells. Figure 4 shows an induction of MDM2 protein in cisplatin treated cells that had been transfected with the R213Q mutant. No induction of MDM2 protein was observed in cells that had been transfected with another p53 mutant that is unable to up-regulate MDM2 (the Y234H mutant). This result shows that the R213Q mutant is able to up-regulate MDM2 during a genotoxic stress response. We also performed a Northern blot and found that the levels of bax or p21 RNA do not increase to a greater extent in R213Q transfected cells versus Y234H or vector alone transfected cells (data not shown). Because we do not have a good positive control for these downstream targets in stably transfected cells (wt p53 is not tolerated in stably transfected cells – see Figure 5), we do not know if the R213Q mutant is not functioning on these promoters during a genotoxic stress response or if the signal generated in these cells is not sufficient to activate R213Q binding function on these promoters.

The results presented above raise the possibility that we have identified a tumor-derived p53 mutant of novel function: one that is deficient in its growth inhibitory activities but is able to induce the expression of the growth promoting molecule MDM2. It remains entirely possible however that this mutant is still a potent growth suppressor due to its ability to up-regulate the expression of growth inhibitory targets other than the ones analysed here or perhaps via growth inhibitory activities of p53 that are independent of sequence specific transactivation. To get an initial indication if this mutant retains growth inhibitory activity, we first determined if transfection of this mutant into cells reduces their growth capacity using a colony formation assay. H1299 cells were transfected with R213Q, two p53 mutants that are devoid of detectable transactivation function (H163C and Y234H), R175P, R181L and wt p53. After transfection, cells were grown in the presence of hygromycin (selectable agent used to maintain the presence of the p53 expression constructs in transfected cells) for 2 weeks and the number of visible colonies were counted. When compared to two other tumor-derived p53 mutants that possess no detectable transactivation function, there is a reduction in the number of hygromycin colonies in R213Q transfected cells. The number of colonies in R213Q transfected cells is however much greater when compared to wt p53 transfected cells, or other p53 mutants (R175P and R181L) with partial growth inhibitory activity (Ludwig *et al.*, 1996; Rowan *et al.*, 1996). These results suggest that the R213Q mutant can inhibit the proliferative capacity of cells when overexpressed at super-physiological levels, although not to the same extent as wt p53.

To get an indication if this mutant retains growth inhibitory activity by another method, we employed a p53 toleration assay. It has been known for some time that mutant p53, but not wt p53, can be expressed at elevated levels in stably transfected tumor cell lines (Baker *et al.*, 1990; Johnson *et al.*, 1991). This is presumably due to the fact that transfected mutant p53 has very little negative effect on cell growth and its expression can be maintained at high levels. In contrast, because wt p53 is a potent growth suppressor, transfection of tumor cells with p53 selects for events which lower the expression of this molecule to a level that is no longer growth inhibitory. Because toleration is assessed under conditions of stable expression when the amount of p53 protein produced in the cells is much less and more at a 'physiological' level than during transient expression, it is likely to be a better indicator of the growth suppressive activity of a molecule when compared to a colony formation assay. To determine if transfected cells can tolerate high levels of the R213Q mutant, two cell lines (one null (H1299) and one with a mis-sense mutation (ASPC-1)) were transfected with this mutant, two other p53 mutants that are devoid of detectable transactivation activity (Y163C and Y234H), the R175P and R181L mutants and a wt p53 expression construct. After transfection, cells were grown in presence of hygromycin for 2 weeks. Protein extracts were prepared from pooled colonies of transfected cells and the relative amount of p53 in these extracts was determined by a Western blot. Figure 5 shows a high amount of p53 protein in R213Q transfected cells. The amount of p53 protein measured in both of these cell lines is comparable to that measured in cells that had been transfected with the other p53 mutant constructs that do not possess any detectable transactivation function. Consistent with a deficiency in p53 transactivation function (Ludwig *et al.*, 1996), the R175P mutant was found to be expressed at higher levels than wt p53 in stably transfected cells. The amount of p53 protein measured in R175P stably transfected cells was however not as high as that measured in R213Q stably transfected cells. As expected, the levels of MDM2 protein were also found to be high in R213Q stably transfected cells (Figure 5). These results show that the R213Q mutant can be stably expressed at high levels in transfected cells and suggests that the R213Q mutant lacks growth suppressive activity under conditions of stable expression.

Because the amount of p53 measured in R213Q transfected cells was the same as that measured in p53 mutant lines that are unable to up-regulate MDM2, we

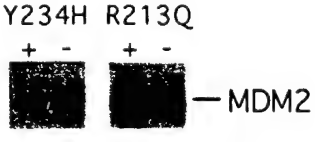


Figure 4 Cisplatin induces MDM2 in stably transfected R213Q p53 mutant cells. Cells from stably transfected R213Q and Y234H p53 mutant expressing cells were either treated with media (−) or media containing 4 μg ml cisplatin for 16 hours (+). After treatment, protein extract was prepared and the level of MDM2 was determined by Western blotting. Equivalent blotting of protein for the Western blots was verified by Ponceau S. staining (data not shown)

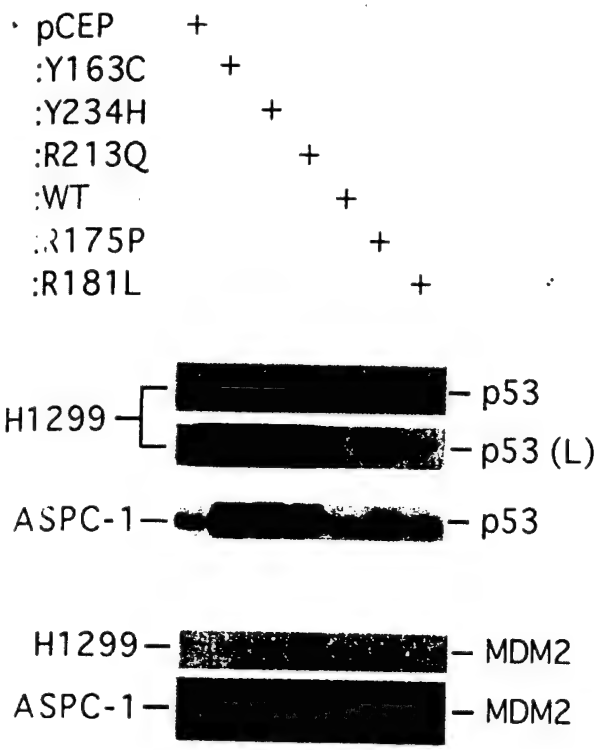


Figure 5 High levels of p53 protein and MDM2 protein are present in R213Q stably transfected cells. H1299 and ASPC-1 cells were transfected with 5 μg each of vector (pCEP), the p53 mutants Y163C, Y234H, R213Q, R175P, R181L and wt p53. H1299 cells were transfected by the calcium phosphate method while ASPC-1 cells were transfected with Lipofectamine. After transfection, cells were cultured in the presence of hygromycin until visible colonies were seen. Cells were then trypsinized and protein extract was prepared from hygromycin resistant cells. The level of p53 and MDM2 protein in these extracts was measured by a Western blot. Ponceau S. staining of the blot after transfer revealed equivalent loading of protein (data not shown)

Table 1 The R213Q mutant is less toxic to transfected H1299 cells than wt p53 or the R175P and R181L mutants

	H163C	*Y234H*	*R213Q*	*R175P*	*R181L*	*wt*
Colonies	63.7±5.7	72.3±9.7	35±3.2	9.3±1.2	13.7±1.7	4±1.6

Cells were transfected with p53 expression constructs in triplicate by the calcium phosphate precipitation method. After transfection, cells were split and cultured in the presence of hygromycin for 2 weeks. The number of visible colonies were then counted

did perform some experiments to determine if this mutant is resistant to MDM2-mediated degradation. However, it appears that this mutant is just as susceptible to MDM2 induced ubiquitination and degradation when compared to wt p53 or other p53 mutants (data not shown). This result was not surprising considering that the level of p53 produced from the CMV promoter is probably in dramatic excess to MDM2 (even when being induced by p53). Thus, it is highly likely that the difference in levels measured between the various p53 constructs is more a reflection of their dissimilar growth control activities and not to their differences in the ability to be degraded by MDM2.

The reasons for why the R213Q mutant is unable to up-regulate the expression of genes analysed here is unclear and obviously requires investigation. We plan to initiate studies that will determine if this mutant can bind to the promoters of genes that it is unable to up-regulate. The p53 binding elements located in the MDM2 promoter is different in both sequence and structure when compared to p53 binding elements that are located in other p53-responsive genes (Zaubermann *et al.*, 1995). It is therefore entirely possible that this mutation is altering the binding of p53 to these promoters and not to the MDM2 promoter. Interestingly, it has been demonstrated that the MDM2–P2 exists in a nucleosome-free state (Xiao *et al.*, 1998) and investigators have speculated that efficient p53 transactivation of some downstream targets may require chromatin remodeling (Zhu *et al.*, 1999). Thus, it is possible that the R213Q p53 mutant may be able to bind to p53 binding elements but is deficient in its ability to recruit proteins that are required for relieving chromatin-mediated repression and/or gene specific transactivation. Determining the reason(s) for why the R213Q is unable to up-regulate the expression of growth suppressive genes may improve our understanding of the mechanisms controlling p53 transactivation specificity.

The p53 growth control pathway can be perturbed by multiple mechanisms in human tumor cells. *p53* gene mutations do give rise to proteins that have no detectable DNA binding activity or transactivation function (el-Deiry *et al.*, 1992; Funk *et al.*, 1992). Interestingly, it is now becoming apparent that mutations can generate a protein with transcriptional activities that are not only distinct from wt p53, but may provide a growth advantage to the tumor cell (so called 'gain of function' mutations). p53 mutants have been identified that can function on the *myc* (Frazier *et al.*, 1998), *BAG-1* (Yang *et al.*, 1999) and *multidrug resistant* gene (Lin *et al.*, 1995) promoters. Considering that MDM2 can alter growth control pathways via p53 independent mechanism when overexpressed (Lundgren *et al.*, 1997; Jones *et al.*, 1998; Sigalas *et al.*, 1996; Sun *et al.*, 1998), it is possible that the R213Q mutant may provide a growth advantage to transformed cells via its ability to up-regulate MDM2 expression alone or in combination with other growth promoting molecules.

The studies presented here and published previously (Crook *et al.*, 1994) show that retention of MDM2 transactivation is not unique to the R213Q mutant. Two tumor derived mutants (R175P and R181L) that possess partial growth suppressive activity are also potent inducers of MDM2 expression. Although these types of mutations may not appear to alter the ratio of MDM2 to negative growth regulators to the same degree as the R213Q mutant, they may do so under certain conditions. For example, both the R175P and R181L mutants have been shown to be functional at inducing cell cycle arrest but not apoptosis and this has been shown to be associated with its ability to up-regulate only a subset of p53-responsive genes (Ludwig *et al.*, 1996; Rowan *et al.*, 1996). These results have led to the speculation that a specific subset of genes are induced by p53 under certain situations and depending on the type of genes that are induced, a p53-mediated cell-cycle arrest or apoptotic-response is initiated. Therefore, signals which induce a p53-apoptotic response in tumors which harbor an apoptotic-defective p53 mutant could result in a dramatic distortion in the ratio of MDM2 to negative growth

regulators. Because MDM2 is involved in the negative regulation of p53 function, we hypothesize that retention of MDM2 promoter transactivation will be a universal feature of tumor derived mutants that retain partial transcriptional activity on growth suppressive targets. If this is the case, MDM2-transactivating p53 mutants may occur in a significant percentage of human cancers and their functional properties need further elucidation.

Acknowledgments

The authors would like to thank W el-Deiry for helpful discussions and D George and M Murphy for critically reviewing the manuscript and M Murphy for help with ChIP. This work was supported in part by grants from NIH (CA70165) and the US Army (DAMD17-97-1-7160).

References

Baker SJ, Markowitz S, Fearon ER, Willson JK and Vogelstein B. (1990). *Science*, **249**, 912–915.

Barak Y, Juven T, Haffner R and Oren M. (1993). *EMBO J.*, **12**, 461–468.

Boyd KE, Wells J, Gutman J, Bartley SM and Farnham PJ. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 13887–13892.

Bull EK, Chakrabarty S, Brodsky I and Haines DS. (1998). *Oncogene*, **16**, 2249–2257.

Cho Y, Gorina S, Jeffrey PD and Pavletich NP. (1994). *Science*, **265**, 346–355.

Crook T, Marston NJ, Sara EA and Vousden KH. (1994). *Cell*, **79**, 817–827.

Dubs-Poterszman MC, Tocque B and Wasylyk B. (1995). *Oncogene*, **11**, 2445–2449.

Duthu A, Debuire B, Romano J, Ehrhart JC, Fiscella M, May E, Appella E and May P. (1992). *Oncogene*, **7**, 2161–2167.

Eischen CM, Weber JD, Roussel MF, Sherr CJ and Cleveland JL. (1999). *Genes Dev.*, **13**, 2658–2669.

el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B. (1992). *Nature Genet.*, **1**, 45–49.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, **75**, 817–825.

Fakharzadeh SS, Trusko SP and George DL. (1991). *EMBO J.*, **10**, 1565–1569.

Frazier MW, He X, Wang J, Gu X, Cleveland JL and Zambetti GP. (1998). *Mol. Cell. Biol.*, **18**, 3735–3743.

Friedlander P, Haupt Y, Prives C and Oren M. (1996). *Mol. Cell. Biol.*, **16**, 4961–4971.

Funk WD, Pak DT, Karas RH, Wright WE and Shay JW. (1992). *Mol. Cell. Biol.*, **12**, 2866–2871.

Hainaut P, Hernandez T, Robinson A, Rodriguez-Tome P, Flores T, Hollstein M, Harris CC and Montesano R. (1998). *Nucleic Acids Res.*, **26**, 205–213.

Haines DS, Landers JE, Engle LJ and George DL. (1994). *Mol. Cell. Biol.*, **14**, 1171–1178.

Johnson P, Gray D, Mowat M and Benchimol S. (1991). *Mol. Cell. Biol.*, **11**, 1–11.

Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW and Vogelstein B. (1992). *Science*, **256**, 827–830.

Jones SN, Roe AE, Donehower LA and Bradley A. (1995). *Nature*, **378**, 206–208.

Jones SN, Hancock AR, Vogel H, Donehower LA and Bradley A. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 15608–15612.

Lin J, Teresky AK and Levine AJ. (1995). *Oncogene*, **10**, 2387–2390.

Ludwig RL, Bates S and Vousden KH. (1996). *Mol Cell. Biol.*, **16**, 4952–4960.

Lundgren K, Montes de Oca Luna R, McNeill YB, Emerick EP, Spencer B, Barfield CR, Lozano G, Rosenberg MP and Finlay CA. (1997). *Genes Dev.*, **11**, 714–725.

Marks DI, Vonderheid EC, Kurz BW, Bigler RD, Sinha K, Morgan DA, Sukman A, Nowell PC and Haines DS. (1996). *Br. J. Hemat.*, **92**, 890–899.

Martin K, Trouche D, Hagemeier C, Sorensen TS, La Thangue NB and Kouzarides T. (1995). *Nature*, **375**, 691–694.

Montagnoli A, Guardavaccaro D, Starace G and Tirone F. (1996). *Cell Growth Differ.*, **7**, 1327–1336.

Montes de Oca Luna R, Wagner DS and Lozano G. (1995). *Nature*, **378**, 203–206.

Murphy M, Ahn J, Walker KK, Hoffman WH, Evans RM, Levine AJ and George DL. (1999). *Genes Dev.*, **13**, 2490–3501.

Pan Y and Haines DS. (1999). *Cancer Res.*, **59**, 2064–2067.

Polyak K, Xia Y, Zweier JL, Kinzler KW and Vogelstein B. (1997). *Nature*, **389**, 300–305.

Roemer K. (1999). *Biol. Chem.*, **380**, 879–887.

Rouault JP, Falette N, Guehenneux F, Guillot C, Rimokh R, Wang Q, Berthet C, Moyret-Lalle C, Savatier P, Pain B, Shaw P, Berger R, Samarut J, Magaud JP, Ozturk M, Samarut C and Puisieux A. (1996). *Nature Genet.*, **14**, 482–486.

Rowan S, Ludwig RL, Haupt Y, Bates S, Lu X, Oren M and Vousden KH. (1996). *EMBO J.*, **15**, 827–838.

Sigalas I, Calver AH, Anderson JJ, Neal DE and Lunec J. (1996). *Nature Med.*, **2**, 912–917.

Sun P, Dong P, Dai K, Hannon GJ and Beach D. (1998). *Science*, **282**, 2270–2272.

Waldman T, Kinzler KW and Vogelstein B. (1995). *Cancer Res.*, **55**, 5187–5190.

Wang Y, Schwedes JF, Parks D, Mann K and Tegtmeyer P. (1995). *Mol. Cell. Biol.*, **15**, 2157–2165.

Wu X, Bayle JH, Olson D and Levine AJ. (1993). *Genes Dev.*, **7**, 1126–1132.

Xiao G, White D and Bargonetti J. (1998). *Oncogene*, **16**, 1171–1181.

Xiao ZX, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR and Livingston DM. (1995). *Nature*, **375**, 694–698.

Yang X, Pater A and Tang SC. (1999). *Oncogene*, **18**, 4546–4553.

Zambetti GP, Bargonetti J, Walker K, Prives C and Levine AJ. (1992). *Genes Dev.*, **6**, 1143–1152.

Zauberman A, Flusberg D, Haupt Y, Barak Y and Oren M. (1995). *Nucleic Acids Res.*, **23**, 2584–2592.

Zhu J, Jiang J, Zhou W and Chen X. (1998). *Cancer Res.*, **58**, 5061–5065.

Zhu J, Jiang J, Zhou W, Zhu K and Chen X. (1999). *Oncogene*, **18**, 2149–2155.

[CANCER RESEARCH 59, 2064–2067, May 1, 1999]

Advances in Brief

The Pathway Regulating MDM2 Protein Degradation Can Be Altered in Human Leukemic Cells[1]

Yi Pan[2] and Dale S. Haines[2, 3]

Barry Ashbee Leukemia Research Laboratories, Hahnemann University Hospital, Philadelphia, Pennsylvania 19102

Abstract

The MDM2 protein regulates the functional activity of the p53 tumor suppressor through direct physical association. Signals that control MDM2 expression are poorly understood but are likely to play an important role in the regulation of p53 activity. We show here that the half-life of MDM2 protein is shorter in proliferating than in quiescent peripheral blood mononuclear cells. We also demonstrate that MDM2 protein half-life is extended in some, but not all, p53 mutant human leukemic cell lines. In at least one of these p53 mutant lines, increased MDM2 protein stability is associated with higher amounts of MDM2 protein. Moreover, we demonstrate that MDM2 protein accumulates to a much greater extent in proteasome inhibitor-treated cells containing unstable MDM2 than in cells possessing stable MDM2. These results demonstrate that MDM2 expression is regulated by events that control the stability of the protein and suggest that the normal regulation of MDM2 turnover can be altered in tumor cell lines.

Introduction

The transforming potential of the MDM2 oncoprotein is activated by overexpression (1, 2), and high levels of MDM2 protein are present in some human tumor cells (3). The primary mechanism by which MDM2 overexpression is thought to induce cellular transformation is through its ability to bind to the p53 tumor suppressor and block p53 activity (4). MDM2 can inhibit p53 activity by binding to the acidic activation domain of p53 (5) and by targeting p53 for degradation via the proteasome (6, 7). Because the transforming activity of MDM2 has been attributed to the overproduction of protein (1, 2), it is important to understand the mechanisms that regulate MDM2 protein expression and how these mechanisms may be altered in human tumor cells. It is well established that p53 is itself a key regulator of *MDM2* transcription (8–11). Besides those signals that induce p53 transcriptional activity (*e.g.*, DNA damage), very little is known about other cellular signals that may regulate MDM2 protein expression. In the work presented here, we have investigated how MDM2 protein stability may be differentially regulated in quiescent and growth stimulated human PBMCs.[4] We have also examined the possibility that the regulation of MDM2 protein stability may be altered in human leukemic cell lines.

Materials and Methods

Cell Culture. All lines were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.). The M8166 (T-cell) line possesses wt *p53* alleles, whereas the CEM (T-cell), HEL (erythroleukemia), Jurkat (T-cell), MOLT4 (T-cell), and Raji (B-cell) lines possess mutant *p53* alleles (12). PBMCs were isolated with Ficoll-Paque (Pharmacia) according to the manufacturer's instructions. Quiescent PBMCs were cultured in complete RPMI 1640 and used for studies immediately after isolation from normal donors. To generate a population of actively proliferating T cells, quiescent PBMCs were seeded at 2×10^6 cells/ml in complete RPMI 1640 supplemented with 1% PHA-M (Life Technologies, Inc.). After 3 days, recombinant IL-2 (Sigma) was added into the media to a final concentration of 1 unit/ml. Cells were counted every other day and maintained at 1×10^6 cells/ml in IL-2-containing media. Once cells possessed a doubling time of ~48 h, they were used for the appropriate studies.

Cycloheximide and MG115 Treatment of Cells. Cells were seeded at 2×10^6 cells/ml in their respective culture medium containing 75 μg/ml of cycloheximide or 10 μM MG115. Cells were harvested at different time points after cycloheximide treatment and pelleted by centrifugation. For Western analysis, cell pellets were stored at −80°C prior to extraction of protein.

Protein Extraction and Western Analysis. Cells were lysed in TENN buffer [50 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 0.5% NP40, and 150 mM NaCl] supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml soy trypsin inhibitor, and 1 μg/ml pepstatin A. Lysates were clarified by centrifugation, and the protein concentration was determined by the Bradford method (Bio-Rad). Samples were then mixed with 4× SDS-PAGE sample loading buffer, boiled, separated on SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. Blots were probed with 0.5 μg/ml p53 (DO-1; Calbiochem), 3 μg/ml MDM2 (IF2; Calbiochem), and actin (0.1 μg/ml, Sigma) antibodies as described previously (12). Signals were visualized by using horseradish peroxidase-conjugated antibodies (sheep anti-mouse for MDM2 and p53, Amersham; goat-anti rabbit for actin, Boehringer Mannheim) and enhanced chemiluminescence (DuPont NEN).

RNA Isolation and RNase Protection. Total RNA was isolated using RNazol B (Tel-Test, Inc.) as per manufacturer's instructions. RNase protection using an MDM2 probe that measures the levels of MDM2-P1 and MDM2-P2 transcripts was performed as described previously (12), except that 7.5 μg of total RNA were used in hybridization reactions.

Results

MDM2 and p53 Half-Life Analysis in Quiescent and Growth-stimulated PBMCs. To determine whether the stability of MDM2 protein may be differentially regulated in quiescent *versus* proliferating cells, the half-life of MDM2 protein was measured in both untreated and PHA/IL-2-treated PBMCs. Freshly isolated PBMCs from normal individuals are comprised predominantly of quiescent T cells (~70%), whereas the treatment of PBMCs with PHA and the culturing of these cells with IL-2 will generate a relatively pure population of cycling T cells. MDM2 protein half-life was measured by determining the level of protein at various time points after treatment with the protein synthesis inhibitor cycloheximide. Interestingly, Fig. 1*a* shows that the level of MDM2 protein declined much more rapidly in cycloheximide-treated, growth-stimulated cells than in unstimulated cells. The half-life of MDM2 in growth-stimulated

Received 12/17/98; accepted 3/19/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grant CA70165 from the National Cancer Institute and by Grant DAMD17-97-1-7169 from the US Army.

[2] Present address: The Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, 3307 North Broad Street, Philadelphia, PA 19140.

[3] To whom requests for reprints should be addressed, at The Fels Institute for Cancer and Molecular Biology, Temple University School of Medicine, 3307 North Broad Street, Philadelphia, PA 19140. Phone: (215) 707-5765; Fax: (215) 707-2102; E-mail: dhaines@unix.temple.edu.

[4] The abbreviations used are: PBMC, peripheral blood mononuclear cell; wt, wild type; PHA, phytohemagglutin; IL, interleukin.

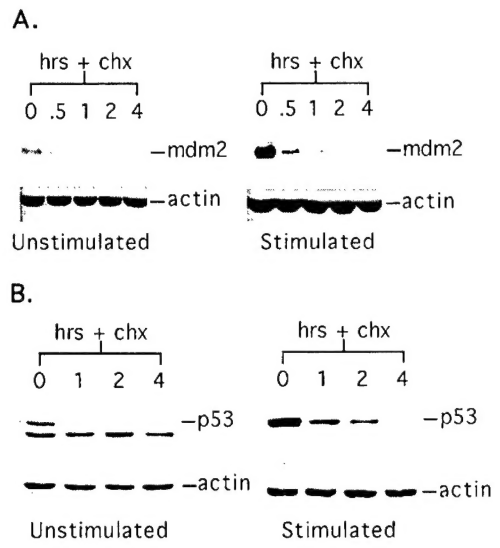


Fig. 1. MDM2 (*A*) and p53 (*B*) half-life in untreated and growth-stimulated PBMCs. PBMCs were obtained from healthy normal individuals and isolated by Ficoll gradients. To generate an active cycling lymphocyte population, PBMCs were stimulated with PHA and then grown in the presence of IL-2 for at least a week until the cells possessed a doubling time of ~48 h. Then, freshly isolated PBMCs and stimulated PBMCs were treated with the protein synthesis inhibitor cycloheximide (at 75 μg/ml) for the indicated period of time. Cells were harvested, extracts were prepared, and the level of either MDM2 (*A*) or p53 (*B*) protein was measured by Western blot analysis as described in "Materials and Methods." Blots were also probed with polyclonal antibodies against actin to show equivalent loading of protein.

cells measured by the method used here (<30 min) is consistent with previous studies that have used radioactive protein labeling methods to measure MDM2 half-life in cell lines (2). Because cycloheximide may alter the expression of other factors that may regulate MDM2 protein stability, we did attempt to measure MDM2 protein stability by radiolabeling and immunoprecipitation as well. Although we were able to measure MDM2 stability in cycling cells by pulse-chase experiments (the half-life of MDM2 protein was found to be very similar to that measured with cycloheximide in these cells), we could not detect MDM2 protein in quiescent cells after radiolabeling and immunoprecipitation (data not shown). This is most likely due to a very low rate of MDM2 synthesis in quiescent cells (12). Therefore, we cannot rule out the possibility at this time that MDM2 half-life is also short in quiescent cells and that cycloheximide is altering the expression of factors that may regulate MDM2 stability in these cells.

MDM2 can regulate the stability of the p53 tumor suppressor through direct physical association (6, 7). To determine whether growth stimulation also modulates p53 protein stability, p53 half-life analysis was performed on untreated and PHA/IL-2-treated PBMCs. Fig. 1*b* shows that there is not a dramatic difference in the half-life of p53 protein measured in stimulated and unstimulated cells. In fact, p53 stability in cycling T cells may even be slightly longer than that measured in quiescent PBMCs. This result significantly reduces the concern that the difference in MDM2 half-life measured in cycling *versus* quiescent PBMCs using cycloheximide is due to a difference in the ability of cycloheximide to block protein synthesis in these two cell populations. It also suggests that the rate of p53 protein degradation is not directly coupled to the rate of MDM2 turnover.

The Half-Life of MDM2 Protein Is Extended in Human Leukemic Cells. The results presented above suggest that MDM2 expression can be regulated by mechanisms controlling MDM2 protein stability. Because MDM2 has oncogenic properties when overexpressed and MDM2 protein levels have been shown to be elevated in a number of different cancers in the absence of increased RNA levels (including in hematological malignancies; Ref. 13), we investigated the possibility that alterations in the regulation of MDM2 protein stability may contribute to high levels of MDM2 protein in human tumor cells. We first performed MDM2 Western blot analysis on a series of leukemic cell lines. Although no lines expressed a high level of MDM2 protein (>10-fold above the level of MDM2 protein measured in PHA/IL-2-stimulated PBMCs), we were surprised to find similar amounts of MDM2 protein in wt and mutant p53-containing lines and that the p53 mutant Jurkat line expressed the highest amount of MDM2 protein (Fig. 2*a*). This line expressed six times more MDM2 protein (as determined by densitometric analysis) than that measured in PHA/IL-2-stimulated PBMCs.

The expression profile of MDM2 in leukemic lines was not expected, considering that the majority of these lines possess mutant p53 and should possess low amounts of MDM2-P2 transcripts (12); p53-regulated transcripts that have been shown to be translated at high efficiencies *in vitro* and in cells in culture (14, 15). To verify the absence of these transcripts in mutant p53 lines, RNase protection analysis was performed. As expected, those lines with mutant p53 did not possess MDM2-P2 transcripts (Fig. 2*b*). Fig. 2*b* also shows that all lines possessed similar amounts of MDM2-P1 transcripts; MDM2 transcripts that are regulated by the p53-independent promoter of the *MDM2* gene. These results raise the possibility that MDM2-P2 transcripts are not translated more efficiently in lymphoid cells. However, it is also conceivable that the half-life of MDM2 protein is longer in some of the lines with undetectable amounts of MDM2-P2 transcripts, but expressing higher or similar level of MDM2 protein as wt p53-containing lines. To address this, the half-life of MDM2 protein was measured in several mutant p53 lines. All lines analyzed possessed similar doubling times, and half-life studies were performed with cells in their logarithmic stage of growth. Fig. 3 shows that the half-life of MDM2 protein was clearly longer in the HEL, CEM, Jurkat, and MOLT4 lines than in the Raji line or in PHA/IL-2-stimulated PBMCs. It is unlikely that the difference in MDM2 half-life measured in the lines is do to an unequal inhibition of protein synthesis by cycloheximide because this compound was found to inhibit incorporation of

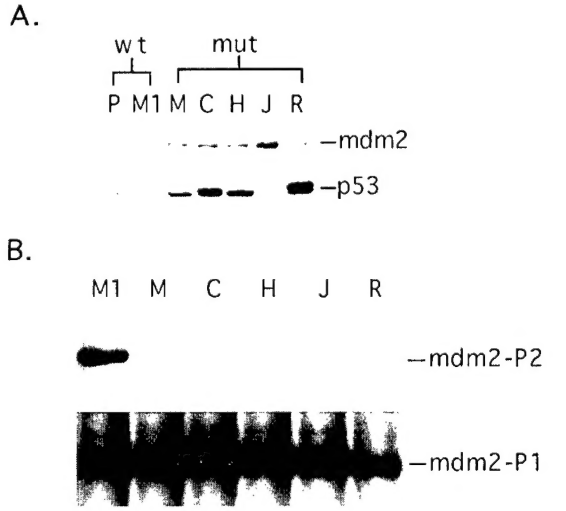


Fig. 2. MDM2 protein and MDM2-P2 transcript levels in human leukemic cell lines. MDM2 and p53 protein levels were measured as described in "Materials and Methods" from the following cell lines: *Lane P,* PHA/IL-2-stimulated PBMCs; *Lane M1,* M8166 T-cell line; *Lane M,* MOLT4 T-cell line; *Lane C,* CEM T-cell line; *Lane H,* HEL erythroleukemia line; *Lane J,* Jurkat T-cell line; and *Lane R,* Raji B-cell line. Ponceau S staining of membranes was performed to verify equivalent loading of protein. High levels of p53 protein were measured in lines possessing missense p53 mutations, whereas undetectable levels of p53 protein were present in the Jurkat line harboring a frameshift p53 mutation. *B,* the levels of MDM2-P1 and MDM2-P2 transcripts was measured in these same lines by RNase protection as described in "Materials and Methods."

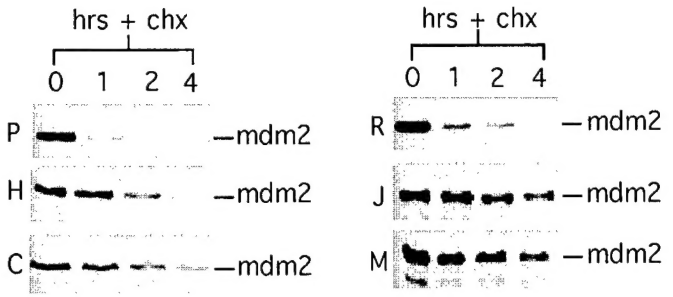


Fig. 3. MDM2 protein stability in human leukemic cell lines. Human leukemic cell lines were treated with the protein synthesis inhibitor cycloheximide (at 75 μg/ml) for the indicated period of time. Cells were then harvested, extracts were prepared, and the level of MDM2 protein in these extracts was subsequently measured by Western blot analysis. Abbreviations used are the same as those used in the legend of Fig. 4. Ponceau S staining of membranes was performed to verify equivalent loading of protein.

[^{35}S]methionine to a similar degree in all lines tested (data not shown).

MDM2 protein has been shown to be degraded via the proteasome (16). The longer half-life of MDM2 protein measured in some of these leukemic lines may be attributed to differences in the way MDM2 protein is degraded by the proteasome. To address this hypothesis, the Jurkat (stable MDM2), MOLT4 (stable MDM2), and Raji (unstable MDM2) lines were treated with the proteasome inhibitor MG115 for varying periods of time, and the level of MDM2 protein was measured after treatment. Fig. 4 demonstrates a greater fold increase in MDM2 protein levels in the Raji line (~5-fold at the 2-h time point as determined by densitometric analysis) than in either the Jurkat or MOLT4 cell lines (both displaying a 2-fold induction of protein at the 2-h time point). This result supports those presented in Fig. 3 that show that the half-life of MDM2 protein is longer in the Jurkat and MOLT4 lines than in the Raji line and suggests that the normal regulation of proteasome-mediated degradation of MDM2 protein can be altered in human leukemic cells.

Discussion

p53 and MDM2 expression is regulated at multiple levels by mitogenic signals. Growth stimulation of human lymphocytes has previously been shown to induce an overall increase in both p53 RNA and p53 protein (17, 18). MDM2 RNA (specifically, those transcripts that are regulated by p53) and MDM2 protein have also been shown to be up-regulated in growth-stimulated cells (12). We show here that the stability of MDM2 protein is regulated by growth stimulation signals as well. Interestingly, we show that MDM2 protein has a shorter half-life in growth-stimulated PBMCs than in quiescent PBMCs. This may appear to be inconsistent with an overall increase in the amount of MDM2 protein. However, considering that MDM2 expression can be regulated at multiple levels, the increase in MDM2 protein measured in stimulated cells is probably due to the fact that growth stimulation induces a greater fold increase in MDM2 transcription and/or translation than turnover of MDM2 protein.

What could be the functional significance for the differential regulation of p53 and MDM2 expression in quiescent *versus* proliferating cells? It is probably not a high priority for a nondividing cell to expend energy required for either the synthesis or degradation of proteins involved in controlling damaged-induced cell cycle arrest. However, an actively proliferating cell needs to possess an increased capacity to respond to the potentially harmful effects of cellular damage. One way to increase the capacity for a p53 response is to increase the amount of latent p53. We propose that this is accomplished by events that regulate p53 and MDM2 RNA production (12, 17, 18), as well as MDM2 degradation (see Fig. 1). The various mechanisms controlling p53 and MDM2 expression are likely to play an important role in generating an optimal ratio of MDM2 and p53 proteins that is needed to maintain high levels of latent p53 protein in actively proliferating cells.

It appears that there are multiple mechanisms that can lead to the overproduction of MDM2 protein in human tumor cells, including *MDM2* gene amplification (3) and enhanced translation of MDM2-P2 transcripts (15). Besides presenting data that suggest that MDM2 protein stability is differentially regulated in quiescent *versus* active cycling cells, we also present data that suggest that the normal regulation of MDM2 protein turnover mediated by the proteasome can be altered in human tumor cells. The half-life of MDM2 protein was found to be extended in a number of leukemic cell lines, and MDM2 accumulated to a much lesser degree in proteasome inhibitor-treated cells harboring stable MDM2 than in treated cells possessing unstable MDM2. In at least one of these lines (the Jurkat line), the increased stability of MDM2 protein appears to contribute to a high amount of protein (a 6-fold greater amount of protein than that measured in normal cycling lymphocytes). It is therefore possible that alterations in the regulation of MDM2 turnover via the proteasome may be another mechanism that can contribute to the overproduction of MDM2 protein in human tumor cells. It is, however, unlikely that stabilized MDM2 is sufficient for the inhibition of p53 function in the cell lines analyzed here because the *p53* gene is mutated in all lines possessing MDM2 with a long half-life. Alternatively spliced variants of MDM2 lacking the p53 binding region have been shown to transform NIH3T3 cells (19), and overexpression of MDM2 has been documented to alter cell cycle control pathways in p53 null cells *in vivo* (20). Recently, it has also been documented that MDM2 overexpression can block the growth-inhibitory activities of TGF-β1 via a p53-independent mechanism (21) and that p53 null transgenic mice that overexpress MDM2 develop a different spectrum of tumors than mice that are only null for p53 (22). Moreover, although rare, alterations in both MDM2 and p53 expression have been detected in primary human tumor cells (23). Therefore, it is conceivable that stabilized MDM2 may provide a selective growth advantage in p53 mutant cells by altering p53-independent growth control pathways. It is, however, possible that alterations in MDM2 turnover do not contribute at all to the transformation process but are a consequence of alterations in other critical growth control molecules that are important in the regulation of MDM2 stability. It has been reported recently that the alternative translation product of the human *CDKN2A* locus (p14^{arf}) is regulated by p53 and can regulate MDM2 stability (24). Thus, MDM2 protein stability may be associated with either p53 or p14^{arf} expression status. Interestingly, both the Jurkat (stable MDM2) and Raji (unstable MDM2) lines possess undetectable levels of p14^{arf} protein (25) and harbor mutant *p53* alleles. It is therefore likely that the stability of MDM2 will not be associated

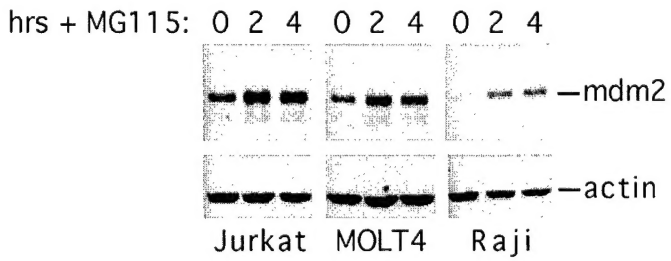


Fig. 4. MDM2 protein levels in MG115-treated cells harboring stable or unstable MDM2. Cells were treated with the proteasome inhibitor MG115 (final concentration, 10 μm) for the indicated period of time. Western blots using either a monoclonal antibody against MDM2 or polyclonal antibodies against actin were performed on protein extracts prepared from untreated and treated cells as described in "Materials and Methods."

simply with p53 or $p14^{arf}$ expression status, and that there are other unknown growth control molecules that are important in the regulation of MDM2 stability that are altered in human leukemic cells.

In summary, our results suggest that MDM2 protein expression is regulated by mechanisms controlling the stability of the protein. It will be of interest for future studies to determine whether MDM2 stability is regulated by signals that not only induce proliferation but also inhibit cell growth (*i.e.,* signals that promote senescence, differentiation, and others). Our data also suggest that mechanisms regulating MDM2 stability can be altered in human tumor cell lines. We did perform half-life studies using primary leukemic cells and found that some patients harbor cells possessing MDM2 with a long half-life (data not shown). However, because we could not control for the proliferative rate of tumor cells derived from the various patients (unlike in cell lines), we do not know if the differences in MDM2 stability measured in primary tumors are due to difference in the proliferative rate of the cells or due to alterations in pathways regulating MDM2 stability. More work will obviously be needed to identify the various factors that play a role in the regulation of MDM2 stability, how alterations in these pathways lead to MDM2 stabilization in cell lines, and whether alterations in the regulation of MDM2 stability occur in primary human tumors.

Acknowledgments

We thank M. Autieri, M. Boyd, and D. George for their critical review of the manuscript.

References

1. Fakharzadeh, S. S., Trusko, S. P., and George, D. L. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J., *19:* 1565–1569, 1991.
2. Finlay, C. A. The *mdm-2* oncogene can overcome wild-type p53 suppression of transformed cell growth. Mol. Cell. Biol., *13:* 301–306, 1993.
3. Leach, F. S., Tokino, T., Meltzer, P., Burell, M., Oliner, J. D., Smith, S., Hill, D. E., Sidransky, D., Kinzler, K. W., and Vogelstein, B. p53 mutation and *MDM2* amplification in human soft tissue sarcomas. Cancer Res., *53:* 2231–2234, 1993.
4. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The *mdm-2* oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, *69:* 1237–1245, 1992.
5. Oliner, J. D., Pientenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature (Lond.), *362:* 857–860, 1993.
6. Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by MDM2. Nature (Lond.), *387:* 299–303, 1997.
7. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. MDM2 promotes the rapid degradation of p53. Nature (Lond.), *387:* 296–299, 1997.
8. Barak, Y., Juven, T., Haffner, R., and Oren, M. MDM2 expression is induced by wild type p53 activity. EMBO J., *12:* 461–468, 1993.
9. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. The p53-mdm-2 autoregulatory feedback loop. Genes Dev., *7:* 1126–1132, 1993.
10. de Oca Luna, R. M., Wagner, D. S., and Lozano, G. Rescue of early embryonic lethality in *MDM2*-deficient mice by deletion of p53. Nature (Lond.), *378:* 203–206, 1995.
11. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. Rescue of embryonic lethality in MDM2-dificient mice by absence of p53. Nature (Lond.), *378:* 206–208, 1995.
12. Bull, E. K., Chakrabarty, S., Brodsky, I., and Haines, D. S. MDM2–P2 transcript levels predict the functional activity of the p53 tumor suppressor in primary leukemic cells. Oncogene, *16:* 2249–2257, 1998.
13. Marks, D. I., Vonderheid, E. C., Kurz, B. W., Bigler, R. D., Sinha, K., Morgan, D. A., Sukman, A., Nowell, P. C., and Haines, D. S. Analysis of p53 and MDM2 expression in 18 patients with Sezary syndrome. Br. J. Haematol., *92:* 890–899, 1996.
14. Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. Regulation of MDM2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. Genes Dev., *8:* 1739–1749, 1994.
15. Landers, J. E., Cassel, S. L., and George, D. L. Translational enhancement of *MDM2* oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. Cancer Res., *57:* 3562–3568, 1997.
16. Cahng, Y. C., Lee, Y. S., Tejima, T., Tananka, K., Omura, S., Heintz, N. H., and Mitsui, J. MDM2 and bax, downstream mediators of the p53 response, are degraded by the uniquitin-proteasome pathway. Cell Growth Differ., *9:* 79–84, 1998.
17. Mercer, W. E., and Baserga, R. Expression of the p53 protein during the cell cycle of human peripheral blood lymphocytes. Exp. Cell Res., *160:* 31–46, 1985.
18. Calabretta, B., Kaczmarek, L., Selleri, L., Torelli, G., Ming, P. M., Ming, S. C., and Mercer, W. E. Growth-dependent expression of human M_r 53,000 tumor antigen messenger RNA in normal and neoplastic cells. Cancer Res., *46:* 5738–5742, 1986.
19. Sigalas, I., Calver, A. H., Anderson, J. J., Neal, D. E., and Lunec, J. Alternatively spliced MDM2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. Nat. Med., *2:* 912–917, 1996.
20. Lundgren, K., Montes de Oca Luna, R., McNeill, Y. B., Emerick, E. P., Spencer, B., Barfield, C. R., Lozano, G., Rosenberg, M. P., and Finlay, C. A. Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. Genes Dev., *11:* 714–725, 1997.
21. Sun, P., Dong P., Dai, K., Hannon, G. J., and Beach, D. p53-independent role of MDM2 in TGF-β1 resistance. Science (Washington DC), *282:* 2270–2272, 1998.
22. Jones, S. N., Hancock, A. R., Vogel, H., Donehower, L. A., and Bradley, A. Overexpression of MDM2 in mice reveals a p53-independent role for MDM2 in tumorigenesis. Proc. Natl. Acad. Sci. USA, *95:* 15608–15612, 1998.
23. Cordon-Cardo, C., Latres, C., Drodnjak, M., Olivia, M. R., Pollack, D., Woodruff, J. M., Marechal, V., Chen, J., Brennan, M. F., and Levine, A. J. Molecular abnormalities of *MDM2* and *p53* genes in adult soft tissue sarcomas. Cancer Res., *54:* 794–799, 1994.
24. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. The alternative product from the human *CDKN2A* locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J., *17:* 5001–5014, 1998.
25. Della Valle, V., Duro, D., Bernard, O., and Larsen, C-J. The human protein $p19^{arf}$ is not detected in hemopoietic human lines that abundantly express the alternative B transcript of the *p16*^{INK4A}*/MTS* gene. Oncogene, *15:* 2475–2481, 1997.